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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Centrosomes are essential organelles that organize mitotic spindles for chromosome segregation and assemble interphase microtubules for controlling cell shape/polarity. All these functions are disrupted in carcinomas, implicating centrosomes in tumorigenesis. We and another group were first to discover that centrosomes were abnormal in most malignant human carcinomas. The work accomplished in this proposal provides evidence for a causative role for centrosomes in prostate cancer. We identified centrosome defects in pre-invasive prostate lesions, showed that they increased with increasing tumor aggressiveness and established a tight link between centrosome defects and genetic instability. We artificially induced centrosome defects in prostate tumor cells by ectopic expression of the centrosome protein pericentrin and induced oncogenic features (hyperproliferation, aneuploidy, modified cell shape/polarity, abrogated mitotic checkpoint). We identified additional centrosome genes that induce aneuploidy when disrupted and novel mechanisms for chromosome missegregation in centrosome-defective tumor cells. We propose a new paradigm for prostate cancer progression. Genetic instability generated by centrosome defects could accelerate accumulation of alleles carrying pro-oncogenic mutations and loss of alleles containing wild-type tumor suppressor genes, thus accelerating genomic changes characteristic of carcinoma. Our results have potential in prostate cancer prognostics (centrosome defects in PIN) and therapeutics (centrosomes, pericentrin, other centrosome proteins as targets).

14. SUBJECT TERMS:
centrosomes, mitotic spindles, aneuploidy, chromosomes, prognostic, diagnostic, therapeutic, drug, PIN, precancer, checkpoint, prostate

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REPORT BODY: INTRODUCTION

References notated by asterisks (*) represent publications related to work in this proposal.

Centrosomes are essential organelles that control a multitude of cellular functions (see *[1]). They are critical elements in the organization of the mitotic spindle and the accurate segregation of chromosomes during mitosis. They also control cell shape and cell polarity, which are fundamental properties of epithelial gland organization. We (and another group) were first to discover that centrosomes were structurally and numerically abnormal in most malignant human carcinomas *[2]. This striking observation has important implications for cancer progression since it suggests that centrosome defects might contribute to cytologic anaplasia and genomic instability that so often accompany these advanced cancers.

REPORT BODY: RESEARCH ACCOMPLISHMENTS

Support for a role for centrosome defects in the development of prostate cancer comes from work accomplished in this proposal. In this progress report, we provide data showing that we have achieved many of the stated objectives and made an unexpected discovery. Below we provide an outline our accomplishments as well as a more detailed explanation of the results and their significance.

Tasks completed:

- Task 1: Identified centrosome defects in a significant number of precancerous lesions *[3].
- Task 1: Identified relationship between genetic instability and centrosome defects in precancerous lesions *[3].
- Task 1: Determined that centrosome defects increase with increasing Gleason grade *[4].
- Task 2: Discovered that pericentrin induces oncogenic features in prostate cells *[4].
- Task 2: Identified novel mechanisms for chromosome missegregation through centrosome dysfunction.
- Task 3: Identified several centrosome proteins that induce genetic instability when silenced by siRNA.

Tasks still underway (funded by no cost extension):

- Task 1: Do centrosome defects in PIN lesions predict bad tumor behavior?
- Task 2: Do pericentrin-expressing cells form induce/exacerbate tumor formation in mice?

We show that centrosome defects, cytologic anaplasia and genomic instability--all features shared by prostate tumor cells--can be artificially induced in nontumor cells and exacerbated in prostate tumor cells by elevating the levels of a single centrosome protein called pericentrin *[4,5]. Based on this and other observations, we proposed a model that views centrosome dysfunction as a critical contributor to prostate cancer development and progression. Consistent with this idea is the known role of centrosomes in nearly all of functions modified during prostate tumor progression from clinically indolent forms (majority) to clinically aggressive forms (minority) [1]. Further support for a role for centrosomes in tumorigenesis comes from another major observation made during the course of this work--that centrosome defects are not only present in aggressive prostate tumors, but also occur in early prostate cancer (low Gleason grade) and in a fraction of "precancerous" (noninvasive) lesions *[3]. Moreover, we show that centrosome defects occur together with genetic instability at this very early stage of prostate cancer providing support for a centrosome-mediated induction of aneuploidy that could contribute to prostate tumor progression *[3]. We used gene silencing methods (RNAi/siRNA) [6] to identify other centrosome proteins that induce aneuploidy and we are now checking these for changes during tumorigenesis. A final aspect of the work that is still underway is to determine whether centrosome defects in PIN lesions are prognostic indicators for aggressive tumor behavior. We had difficulty obtaining sufficient quality material to complete this task, but are now in a position to do so.

In summary, our data are consistent with the idea that centrosome defects and elevated pericentrin levels contribute to rather than result from the tumorigenic process. This work is highly relevant to prostate cancer biology because it has the potential to uncover a unique pathway for prostate cancer progression that may also be involved in the genesis of prostate cancer [7,8]. Moreover, elucidation of the mechanisms and molecules such as pericentrin and other novel aneuploid-inducing centrosome proteins identified during the course of this study, may identify new and powerful prognostic markers as well as novel cancer-specific therapeutic targets for clinically aggressive prostate cancer, the form of prostate carcinoma that is clinically most critical in terms of diagnosis, treatment and health care expenditure.

A more detailed description of the first 4 accomplishments listed above (tasks completed) can be found in manuscripts included in the appendices. The 5th accomplishment was made through the development of technology to image chromosome segregation in living normal and tumor cells using GFP histone 2B. Two novel observations were made. First, we found that dividing tumor cells with only a single centrosome (2 is normal) were able to organize a bipolar spindle by using the plasma membrane to form a spindle pole. To our knowledge, this has never been reported in any human cell. Second, we found that chromosomes in tumor cells segregated normally when bipolar spindles were formed but lost their attachment to the spindle if multipolar spindles were organized. This indicated that the geometry of the chromosomes relative to the centrosomes or forces thereby exerted, are important for segregating chromosomes. We do not believe that this has ever been suggested as a mechanism for loss of chromosomes and subsequent formation of micronuclei. The 6th accomplishment was the identification of 6 other centrosome genes that induce aneuploidy using a screen involving gene silencing. These genes are now being investigated as potential contributors to the prostate cancer phenotype. We are testing for changes in their levels during prostate cancer progression. With this new and powerful strategy in operation, we will not examine centrosome fractions from normal and tumor cells to identify changes in centrosome proteins as originally planned.

REPORT BODY: REPORTABLE OUTCOMES.

- **Manuscripts related to work done during the course of this proposal (5 published, 3 in revision, 3 in preparation):**

1. Zimmerman, W. and Doxsey, S. Interaction between pericentrin and the gamma tubulin ring complex is required for microtubule organization, spindle integrity, cell cycle progression and genetic stability. (in preparation).
2. Rosa, J, Wahl, G. and Doxsey, S. Tumors in mice with the p53 xx mutation have significantly greater centrosome defects and genetic instability than in p53 null mice. (In preparation).
3. Pihan, G. and Doxsey, S. Mutations and aneuploidy in the origins of cancer: Strange bedfellows or a marriage made in heaven? (commissioned by Cancer Cell)
4. Chen, D., Purohit, A., Doxsey, S. and Newton, A. Pericentrin anchors PKC bII at centrosomes and when disrupted induces multipolar spindle formation and aneuploidy. (in preparation).
5. Pihan, G. Zhou, Y., Wallace, J., Zhou, Y. and Doxsey, S. Centrosome abnormalities and genetic instability occur together in precancerous lesions. (in revision for Cancer Research)
6. Gromley, A., Jurczyk, A., Sillibourne, J., Halilovic, E., Powers, C., Craig., R. Blomberg, M., Mogensen, M. and Doxsey, S. A novel protein of the maternal centriole involved in cytokinesis and cell cycle progression. (in revision for Nature Cell Biology)
7. Doxsey, S. Duplicating dangerously: linking centrosome duplication and aneuploidy. Molecular Cell 10, 1-2, 2002.
8. Doxsey, S.J. Re-evaluating centrosome function. Nature Reviews in Molecular Biology 2, 688-699, 2001.
9. Pihan, G., Purohit, A., Wallace, J., Liotta, L. and Doxsey, S. Centrosome defects can account for the cellular and genetic changes that accompany prostate cancer progression. Cancer Res. 61, 2212-2219 (2001) ***(see editorial in Science 292, 426-429, 2001).***
10. Doxsey, S.J. Centrosomes as command centers for cellular control. Nat. Cell Biol. 3, E1-E3, 2001.

11. Tynan, S., Purohit, A., Doxsey, S. J. and Vallee, R. A distinct subclass of cytoplasmic dynein containing light intermediate chain 1 is specific for pericentrin binding. J. Biol. Chem. 275, 32763-32768, 2000.

- **Presentations Related to Prostate Cancer (36 total, presented by S. Doxsey, 2000-2003):**

In 2000: 3/00, Symposium on Colon Cancer Development and Progression, Montreal, Canada; 3/00, American Heart Association Research Conference, Dallas, TX; 11/00 Massachusetts General Hospital, Boston, MA; 11/00 University of Minnesota, Dept. Cell Biology, Minneapolis, MN; 12/00 American Society Cell Biology, "Spindle pole duplication and function".
In 2001: 1/01, University of California, San Diego, CA; 1/01, Fred Hutchison Cancer Center, Seattle, WA (host, Brian Reid) ; 4/02, University of Virginia; 5/01, Armed Forces Institute of Pathology, National Institutes of Health, Washington, D.C.; 5/01, Dana Farber Cancer Research Center, Boston, MA; 6/01, Gordon Research Conference "Centrosome Assembly"; 7/01, Marine Biological Laboratories, Woods Hole, MA; 8/01, FASEB Res. Conf., "Nuclear Structure and Cancer", Saxtons River, VT; 10/01, AACR Special Conference on Cancer and Chromosomal Organization, "Centrosomes and Cancer", Palm Desert, CA.; 12/01, American Society of Cell Biology, Washington, D.C., "Centrosome-Anchored Regulatory Pathways".
In 2002: 2/02 Northwestern University; 4/02, AACR Sunrise Session: "Centrosomes and Cancer"; 4/02, Amherst College; 4/02 Columbia University, NY, NY; 4/02, National Cancer Institute Workshop on "Mitotic Checkpoints and Cancer"; 6/02, Louisiana State University; 9/02, Symposium "Centrosomes" Heidelberg< Germany; 9/02, Cytokinetics SAB; 9/02 CapCURE (poster); 9/02, CNIO Cancer Conference, "Cell Cycle and Cancer", Madrid, Spain; 10/02, University of Iowa, Iowa City, IA; 11/02, Penn State University, Hershey, PA; 12/02, Carnegie Institute, Washington, D.C.; 12/02, American Society of Cell Biology, San Francisco, CA, "Centrosomes and Cancer"; 12/02, American Society of Cell Biology, San Francisco, CA, "siRNA to target centrosome genes in normal and cancer cells".
Planned for 2003: 4/03, "Plenary Lecture", USGEB Meeting, Davos, Switzerland, "Centrosomes and Cancer"; 4/03, Worcester Polytechnic Institute; 4/03, University of Pittsburg; 5/03, Wistar Institute, University of Pennsylvania; 6/03, FASCEB meeting; "Nuclear Structure and Cancer"; 07/03, Clark University, Worcester, MA.

Abstracts Related to Prostate Cancer (33 during 2000-2002).

- **Patents.**
  - 1. Cancer Detection by Centrosome Abnormality (U.S. #5,972,626).
  - 2. Centrosome Defects in precancerous lesions as tools for tumor diagnostics, prognostics and treatment (U.S. patent pending; UMass docket#:UMMC 02-33)
  - 3. Novel centrosome proteins involved in cytokinesis and cell cycle control (U.S. patent pending; UMass docket #: UMMC 02-23)
  - 4. Centrosome protein-induced cell cycle arrest as a potential cancer therapeutic (patent pending)

- **Clinical Translational Research.** We ultimately hope to develop prognostic tests and therapeutics for detection of centrosome defects in PIN and for the treatment of aggressive prostate cancer, respectively.

- **Development of Permanent Cell Lines (Prostate Tumor and Nontumor Lines):**

1. HA-pericentrin-expressing cells (tetracycline-inducible) to study oncogenic effect of pericentrin. We constructed 3 tumor lines (PC-3, DU-145, LN-Cap).
2. GFP-histone (H2B)-expressing cells for studying segregation of GFP-labeled chromosomes in living cells: PC-3, DU-145, LN-Cap.

- **Funding obtained based on this work. 1.** Received funding from Department of Defense for a postdoctoral fellow in Doxsey laboratory to work on centrosomes in breast cancer (Aruna Purohit, $50K, 1 year, 2000, Concept Award, BC996519). **2.** Received funding from Department of Defense for a postdoctoral fellow in Doxsey laboratory to work on centrosomes in breast cancer (Keith Mikule, $171K, 3 years, 2003-2006, Postdoctoral Traineeship Award, BC021594). **3.** Received funding from Department of Defense for a predoctoral fellow in Doxsey laboratory to work on centrosomes in breast cancer (Agata Jurczyk, $90K, 3 years, 2003-2006, Predoctoral Traineeship Award, BC020172). **4.** Received funding from The Hershey Family Foundation/Prostate Cancer Walk Committee to intitiate studies on "Differentiation Therapies Based on Inhibiting Centrosome Genes" (1 year, $30K, 2003).
- **Promotions obtained based on research supported by this award.** The P.I. (SJD) was promoted to Associate Professor (7/99) based in part on work accomplished in this proposal and is currently being promoted to Full Porfessor based in part on this work. Dr. Aruna Purohit was promoted to Instructor based on her work on prostate cancer (2000).
- **Reviewer**: Prostate Cancer Review Panel (1997-present) and Breast Cancer Review Panel (1999-present); 2002: ad hoc reviewer, NIH.

REPORT BODY: CONCLUSION.

We believe that our work on centrosome dysfunction will have a significant impact on our understanding of prostate cancer progression and etiology. It also has the potential to improve our ability to detect and treat the more aggressive and devastating forms of this disease. One compelling reason for this assertion is that our hypothesis examines a fundamentally different and unexplored mechanism for prostate cancer development and progression: that centrosome defects cause genetic instability and cytologic anaplasia, and thus underlie the genesis of malignant disease. For this reason, the reviewers of the original proposal described it as unique and highly innovative. Insights gained from this approach should yield novel information on cellular processes, structures (centrosomes) and molecules (pericentrin) that have the potential to serve as therapeutic targets and prognostic indicators of malignant disease. The discovery that centrosome defects are present in precancerous lesions of the prostate and the identification of a tumor-specific centrosome abnormality indicates that centrosome defects may serve as prognostic indicators of malignant disease. The presence of centrosome defects at the earliest disease stages also suggests that centrosomes may play an active role in the tumorigenic process and thus, may serve as prime therapeutic targets. We hope to complete the analysis of PIN lesions to determine if centrosome defects at this stage serve as prognostic indicators of aggressive disease. Pericentrin may be a novel molecular target for prostate cancer therapeutics since artificial elevation of pericentrin in normal prostate cells induces a tumor-like phenotype and since pericentrin levels are specifically elevated in tumors and precancerous lesions. Within the next year we will test whether pericentrin-expressing cells develop or exacerbate tumor formation in vivo. Other proteins identified in the siRNA screen of centrosome protein function may also serve as prime targets for cancer therapeutics. In conclusion, we have produced compelling data in support of our unique centrosome-based model for prostate cancer progression. We believe this research will provide novel and more discriminating tools for prostate cancer prognosis and treatment.

REFERENCES.

1. Doxsey, S. J. Re-evaluating centrosome function. *Nature Reviews in Molecular Biology* **2**, 688-699 (2001).
2. Pihan, G., Purohit, A., Knecht, H., Woda, B., Quesenberry, P. and Doxsey, S. Centrosome defects and genetic instability in malignant tumors. *Cancer Research* **58**, 3974-3985 (1998).
3. Pihan, G. A., Wallace, J., Zhou, Y. & Doxsey, S. Centrosome abnormalities and chromosome instability occur together in pre-invasive carcinomas. *Cancer Research* **in revision** (2003).
4. Pihan, G., Purohit, A., Wallace J., Malhotra, R., Liotta, L. and Doxsey, S. Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression. *Cancer Res* **61**, 2212-9. (2001).
5. Doxsey, S. J., Stein, P., Evans, L., Calarco, P. & Kirschner, M. Pericentrin, a highly conserved protein of centrosomes involoved in microtubule orgainzation. *Cell* **76**, 639-650 (1994).
6. Elbashir, S. M. et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494-8. (2001).
7. Doxsey, S. Duplicating dangerously: linking centrosome duplication and aneuploidy. *Mol Cell* **10**, 439-40. (2002).
8. Pihan, G. A. & Doxsey, S. Mutations and aneuploidy in the origins of cancer: Strange bedfellows or a marriage made in heaven? *Cancer Cell* **commissioned for early 2003**.

APPENDICES TO FOLLOW

Title: Centrosome abnormalities and chromosome instability occur together in pre-invasive carcinomas.

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Departments of *Pathology and †Molecular Medicine, University of Massachusetts Medical School, Worcester, MA.

Running title: centrosomes in carcinoma in situ.

Keywords: Centrosomes, chromosome instability, in-situ carcinoma

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Abstract.

Centrosomes play critical roles in processes that ensure proper segregation of chromosomes and maintain the genetic stability of human cells. They contribute to mitotic spindle organization and regulate aspects of cytokinesis and cell cycle progression. We and others have shown that centrosomes are abnormal in most aggressive carcinomas. Moreover, centrosome defects have been implicated in chromosome instability and loss of cell cycle control in invasive carcinoma. Others have suggested that centrosome defects only occur late in tumorigenesis and may not contribute to early steps of tumor development. To address this issue, we examined pre-invasive human carcinoma in-situ lesions for centrosome defects and chromosome instability. We found that a significant fraction of precursor lesions to some of the most common human cancers had centrosome defects including in situ carcinomas of the uterine cervix, prostate and female breast. Moreover, centrosome defects occurred together with mitotic spindle defects, chromosome instability and high cytologic grade. Since most pre-invasive lesions are not uniformly mutant for p53, the development of centrosome defects does not appear to require abrogation of p53 function. Our findings demonstrate that centrosome defects occur concurrently with chromosome instability and cytologic changes in the earliest identifiable step in human cancer. Our results suggest that centrosome defects may contribute to the earliest stages of cancer development through the generation of chromosome instability. This, together with ongoing structural changes in chromosomes, could accelerate accumulation of alleles carrying pro-oncogenic mutations and loss of alleles containing wild-type tumor suppressor genes and thus accelerate the genomic changes characteristic of carcinoma, the most prevalent human cancer.

Introduction.

Chromosomal instability or CIN is the most common form of genetic instability in human cancer and is thought to be caused by continuous chromosome missegregation during mitosis **(1, 2)**. Together with structural chromosome changes caused by chromosome breakage and missrepair, CIN is thought to be important to promote the Darwinian genomic evolution characteristic of cancer whereby protooncogene mutations accumulate and normal alleles of mutated tumor suppressor genes are lost **(2-4)**. In fact, loss of heterozygocity in cancer primarily affects whole chromosomes or large chromosomal domains suggesting that it results from gains or losses of entire normal or rearranged chromosomes **(5)**. CIN is thought to facilitate the inexorable evolution of cancers toward cellular states that support tumor cell growth, dissemination and resistance to therapy **(1-3, 6, 7)**. A common element in the chain of events associated with loss of fidelity in chromosome segregation is centrosome dysfunction (for review, see **(7-12)**).

Centrosomes are the primary microtubule-organizing centers in animal cells. They contribute to the organization of microtubule spindles in mitosis and appear to control progression through cytokinesis and entry into S phase **(9, 13-15)**. Our laboratory and another first detected centrosome defects in aggressive carcinomas of multiple origins **(2, 16)**. Several subsequent studies confirmed these observations and extended them to other tumor types and animal models **(17-22)**. The discovery of centrosome defects in essentially all carcinomas sparked interest in this organelle as a global contributor to the development and progression of tumors that exhibit genetic instability **(2, 8-11, 23)**. The established role of centrosomes in organizing mitotic spindles suggested a model in which tumor cells with multiple centrosomes

organize mulitpolar spindles that missegregate chromosomes and contribute to genetic instability. This phenomenon could occur in diploid cells or in cells that previously failed in cell division to create polyploid cells with supernumerary centrosomes **(24)**. Despite the occurrence of centrosome defects in most common human cancers and their known role in the assembly of mitotic spindles and chromosome segregation, a role for centrosomes in the earliest steps of human tumor development has not been well established.

Recent results from our laboratory have shown that centrosome defects and genetic instability occur in some low grade prostate tumors suggesting that they are present prior to development of aggressive tumors **(21)**. Moreover, overexpression of some centrosome-associated proteins, including pericentrin, TACC, polo and aurora **(21, 24-28)** induce tumor-like features. Centrosome defects have also been observed during the early stages of tumor development in a rat mammary carcinogenesis model **(29)** suggesting that centrosome defects may also occur in pre-invasive human tumors. A recent study of invasive human carcinoma showed that centrosome abnormalities occurred in some pre-invasive breast lesions **(20)**. The authors analyzed seven cases in a single tissue (breast) and reported on one parameter of centrosome defect (size) but did not examine the relationship between centrosome defects and CIN in the pre-invasive lesions. It is important to perform a comprehensive analysis of centrosome defects in pre-invasive lesions for several reasons. A comparative analysis of pre-invasive lesions from tissues with different propensities to develop aggressive cancers may provide important information about the role of centrosomes in the development and progression of cancer. This type of analysis could also identify centrosome defects as a universal diagnostic indicator of most, if not all carcinomas. The presence of centrosome defects in pre-invasive lesions may also provide a prognostic marker for tumor development,

especially in prostate cancer where the relationship of pre-invasive lesions to aggressive cancer is unclear. .

In this study, we analyzed 116 pre-invasive lesions from three different human tissues (breast, cervix, prostate). We show that centrosome defects occur in all tissue tissues and that they co-segregate with other tumor-like features associated with centrosome dysfunction including spindle abnormalities, cytologic changes and chromosomal instability **(2, 21)**.

Materials and Methods.

Immunohistochemical staining and analysis. Formalin-fixed paraffin-embedded tissue from carcinoma in situ of the uterine cervix, female breast and male prostate was selected from the files of the Pathology Department at UMass Memorial Health Care. Samples were immunostained with pericentrin antibodies as described **(2, 21, 27).** Standard histopathologic criteria were applied to newly prepared hematoxylin and eosin stained sections to confirm the presence of carcinoma in situ in the specimen **(30)**. Centrosomes were considered abnormal if they had a diameter greater than twice the diameter of centrosomes present in normal epithelium within the same section, if the ratio of their greatest and smallest diameter exceeded 2 or if there were more than two centrosomes in more than 5% of the cells examined **(21)**. γ-tubulin was chosen to stain mitotic spindles in archival formalin fixed paraffin embedded tissues because it decorates spindle poles (Pihan, unpublished) while a large fraction of α and β tubulins are cytoplasmic and obscure the spindle microtubule signal (Pihan, unpublished observations). Multipolar mitoses, an obvious consequence of supernumerary centrosomes, are common in carcinoma cell lines with abnormal centrosomes as we and others have previously shown **(2, 22, 31, 32)**.

CIN analysis. Tissue sections parallel to those used for pericentrin immunohistochemistry were used to stain centromeres of chromosome 1 and 8 **(2)**. Briefly, after de-paraffinization, sections were co-denatured with biotinylated centromeric probes specific for chromosomes 1 or 8 and hybridized overnight at 37°C in a Hybrite oven (Vysis, Chicago, IL) in the hybridization buffer recommended by the probe manufacturer. After appropriate stringency washes sections were placed on the automatic immunostainer and an ABC/DAB protocol

similar to the one used above for immunohistochemistry was used to reveal the hybridized probe. Nuclei were lightly counterstained with hematoxylin. For quantitative analysis, the number of hybridization signals in 100 to 200 nuclei from in-situ carcinoma and morphologically normal adjacent epithelium was recorded **(2)**. Using these probes it has been shown that normal diploid tissue has 10-15% cells with more than 3 signals per nucleus **(2, 33)**. In tissue sections some nuclei are truncated leading to artificially increased numbers of diploid cells with apparently less than two signals per nuclei. For this reason, we primarily computed signal gains (greater than two) and not apparent losses. We also separately analyzed cells with only one copy of a given chromosome. Due to limitations imposed by truncation artifacts, we did not attempt to obtain an absolute measure of chromosome instability in sections, as it can be done on cell lines **(2, 6)**. Rather, we defined tumors with likely aneuploidy/CIN as those in which the fraction of nuclei with more than two signals exceeded 20% **(33)** and used this measurement as an index of chromosome instability/aneuploidy. Cells with only one chromosome were recorded and discussed separately.

Results.

Centrosome defects are present in pre-invasive cancerous lesions. Using antibodies to the centrosome protein pericentrin **(35)**, we examined centrosomes in carcinoma in-situ of the uterine cervix (CIC), female breast (DCIS) and prostate (PIN) as described **(2, 21)**. Several distinct centrosome abnormalities were detected in these lesions, including supernumerary centrosomes (Fig.1 B arrowheads), abnormally shaped centrosomes, such as elongated or corkscrew forms (Fig.1 D, F) and centrosomes of larger diameter than those in normal epithelium within the same tissue section (Fig.1 B, D). Thirty to seventy two percent of all precancerous lesions had abnormal centrosomes (Fig.2 A-C) while such abnormalities were rarely, if ever, detected in nontumor cells (Fig.2 A-C). Centrosome defects were more prevalent in DCIS and CIC lesions than in PIN lesions. This difference was consistent with differences in histological, cytological and genetic features of these lesions. For example, DCIS and CIC show a high degree of nuclear atypia, cytologic disarray, loss of cell polarity and genetic instability. In fact, on cytologic features alone, they are often indistinguishable from invasive breast and cervical cancers **(36, 37)**. In contrast, PIN lesions show remarkable preservation of cell polarity and glandular architecture and can only be distinguished from normal glands by subtle changes in nuclear and nucleolar features. Similar levels of centrosome defects in pre-invasive lesions were identified using antibodies to γ-tubulin, another core protein of the centrosome (data not shown).

The incidence of centrosome defects increases with higher histologic grade of in-situ carcinomas. In-situ carcinomas of different histologic/cytologic grade differ in their associated risk of progression to invasive carcinoma **(36, 38, 39)**. We observed a higher

incidence of centrosome defects in the higher grades of all three precancerous lesions (Fig. 3). The increase in centrosome abnormalities was greater in the lesions associated with a higher propensity to evolve into invasive carcinoma, and is consistent with a model where centrosomes contribute to the cytologic and genetic changes that occur during progression of precancerous lesions.

Mitotic spindle abnormalities are frequent in carcinoma in situ. One expected consequence of supernumerary centrosomes in mitotic cells was the development of multipolar mitotic spindles **(2, 27)**. To identify abnormal spindles, we stained sections with γ-tubulin, which provided the best marker for spindle poles in our immunohistochemical procedure (Fig. 4, see Materials and Methods). The total number of mitotic figures was generally low. The percentage of samples that contained spindles was 74% (29/39, CIC), 35% (12/34, DCIS) and 0% (0/42, PIN). The low incidence of spindles in PIN lesions is likely the result of delayed fixation of these tissues and the relatively slow growth of prostate tumor cells compared with the other in situ lesions. Of the tumors with spindles, 75% (9/12) of DCIS and 34% (10/29) of CIC had at least one abnormal spindle (Fig. 4H and G). Defective spindles included multipolar spindles (3 or more poles, Fig. 4 B, D, F), multiple bipolar spindles in single cells (Fig. 4E) and asymmetric bipolar and multipolar spindles (Fig.4D, F). In lesions with 10 or more spindles, a number chosen to avoid the inherent bias introduced in data by low spindle counts, the average number of multipolar spindles was 10-16% (Fig. 4I). Monopolar spindles were also detected but they could not be authenticated due to the compounding effect of truncation artifacts induced by tissue sectioning. Mitotic figures were infrequently observed in normal epithelium adjacent to in situ lesions, a likely consequence of the low mitotic rate of these tissues. When present, these spindles were normal (symmetric,

bipolar, n=4, data not shown). The absence of spindle abnormalities in normal tissues was consistent with our previous results in nontumor tissues (5, 20) and was confirmed in another epithelial tissue with a high proliferative rate. In samples of celiac sprue, a form of intestinal malabsortion in which the intestinal epithelium has increased mitotic activity do to increased rates of mucosal regeneration, we never observed abnormal mitoses (n= 45). Taken together, these data indicated that spindle defects were specific for in situ lesions.

Centrosome defects correlate with CIN in precancerous lesions. Both chromosome instability **(2, 6, 16)** and centrosome defects are common features of epithelial cancers **(11, 16, 21, 31).** We have previously demonstrated a correlation between the extent of centrosome defects and CIN in invasive prostate cancer **(2, 21).** To determine whether a correlation exists between centrosome defects and CIN in carcinoma in situ, we examined consecutive serial tissue sections for these anomalies **(2, 21).** While CIN was observed in many in situ lesions, it was never seen in normal epithelium in the same tissue section (Fig. 5A, C and E). Moreover, in all three in situ carcinomas there was a statistically significant non-random association (Fisher exact test $p < 0.005$) between centrosome defects and CIN (Fig. 5G-I). In fact, most lesions with centrosome defects showed CIN (63-71%, Fig. 5). Conversely, the fraction of cases that lacked centrosome defects lacked CIN (81-95%, Fig. 5). The correlation between centrosome defects and CIN was significant despite the vastly different degrees of centrosome defects between DCIS, CIC and PIN (Fig 2). Interestingly, there were more lesions that had centrosome defects and lacked CIN (~30%) than lesions with CIN that lacked centrosome defects (~10-20%), consistent with a model where centrosome defects precede CIN in the progression of the tumor-like phenotype in precancerous lesions **(2, 9).** Comparison of the fraction of cells with one chromosome signal (hypoploids) across all three precancers showed

that carcinoma in situ invariably contained fewer cells with one chromosome signal than control tissues (DCIS, 28.9 +/- 12 vs. control, 38.1 +/- 10, p=0.023; CIC, 31.2 +/- 12 vs. control 41.3 +/- 9, p=0.0023 and PIN, 24.6+/- 13 vs. control 31.2 +/- 10, p=0.03). In conclusion, in situ lesions have a lower frequency of single chromosome copy number and a higher frequency of multiple chromosome copy number suggesting that cells in these early lesions are mostly polyploid and almost never hypoploid.

Discussion.

Our results demonstrate that centrosome defects are present in a significant fraction of in situ carcinomas of the breast, cervix and prostate. These results extend our previous observations that centrosome defects are present in low-grade tumors and increase in more aggressive carcinomas **(21).** They also expand on other studies showing centrosome defects in a limited number of in situ lesions from human breast and rat tissues **(20, 29).** Because p53 mutations are not universal in these pre-invasive lesions (see below) we conclude that abrogation of p53 function is not a prerequisite for the development of centrosome defects early in tumor development. These observations are consistent with a role for centrosome defects in the establishment of carcinoma and perhaps the progression of early lesions to more aggressive cancers.

Centrosome defects occur frequently in advanced forms of some of the most common human cancers and may contribute to genetic instability by impairing the fidelity of chromosome segregation during mitosis **(1, 3, 8, 9, 11, 16)**. It is currently held that carcinoma in-situ is the immediate precursor of invasive epithelial cancers and that it shares some but not all genotypic and phenotypic characteristic of invasive cancer **(38, 40, 41)**. Our results show that centrosome defects are present at the earliest morphologically recognizable stages of tumor development in some of the most common human cancers. They provide a mechanistic explanation for the commonly observed CIN and aneuploidy observed in most lesions found in human carcinoma in situ and experimental models of carcinogenesis **(33, 42-45)**. These data are consistent with a role for centrosome defects in the generation of genetic instability during the early stages of the tumorigenic process.

Our study also demonstrates that centrosome defects correlate with the histologic/cytologic grade of the in-situ lesion and thus support a role for the centrosome in the induction of the morphologic phenotype characteristic of carcinoma in situ. Centrosomes have been shown to play a role in cell polarity **(46)** shape **(47, 48)** and motility **(47)**, all of which are perturbed in all in-situ cancers examined in this study. Moreover, the presence of mitotic spindle defects in many CIC and DCIS lesions and the co-segregation of centrosome abnormalities with CIN, strongly suggest that centrosome defects have a functional impact in in-situ carcinoma.

Our results are consistent with a role for centrosome defects in the development of aggressive tumors, rather than those that remain benign. This idea is supported by the high prevalence of centrosome abnormalities in lesions with a high rate of progression to high-grade cancer (DCIS and CIC) and the low prevalence of centrosome defects in PIN lesions, the majority of which progresses to low grade invasive cancers. Because DCIS and CIC are usually indistinguishable cytologically from aggressive cancers **(36, 49)** it is believed that they give rise to these aggressive cancers. In contrast, cancers of the prostate are usually low-grade **(50)** consistent with the low-grade appearance of most PIN lesions. These results support our centrosome-mediated model of tumor genesis **(2)** where centrosome defects induce dramatic and persistent changes in chromosome number (CIN) thereby shuffling the genome and allowing selection of the most aggressive phenotypes such as those seen in invasive cancers.

The presence of centrosome abnormalities at the earliest stages of disease may also have the potential to predict evolution of in-situ lesions into high-grade invasive cancers. This

is of particular interest for the management of prostate cancer since the majority of these tumors are biologically low grade, but with time may progress to aggressive form. Currently, these cancers are often treated by prostatectomy because there is no effective prognostic indicator of aggressive disease. If centrosome abnormalities can predict development to high-grade cancer, they would provide a sorely needed surrogate marker for aggressive disease. We are currently testing if centrosome defects correlate with aggressive prostate cancer by examining PIN lesions from patients that subsequently progress to invasive cancer. Based on our previous work showing that the incidence of centrosome defects are higher in more aggressive tumors **(21),** we are hopeful that their incidence will also be higher in precancerous lesions that subsequently progress to aggressive tumors.

An interesting observation made in this study was the presence of low, yet measurable levels of centrosome defects in morphologically normal epithelium adjacent to CIC lesions (Fig. 2A). We speculate that this may be due to the presence of human papilloma virus infection. It is well established that papilloma virus is the cause of nearly all carcinomas of the cervix and is present in all precursor lesions **(51)**. Moreover, it has recently been demonstrated that papilloma virus can rapidly induce centrosome abnormalities in squamous epithelial cells in vitro **(52)**.

Our observations also suggest a mechanism for centrosome-mediated generation of genetic instability in carcinoma in situ. The excellent correlation between centrosome defects, aberrant spindles and CIN indicate that abnormal centrosomes contribute to spindle disorganization, chromosome missegregation and genetic instability in these lesions. These data also suggest that supernumerary centrosomes lead to multipolar spindles and do not

merely coalesce to form bipolar spindles as it has been suggested from work in cell lines **(8, 53)**.

Although our study answers the important question of whether centrosome defects occur in pre-invasive cancers, it also leaves unanswered a number of interesting issues. One of the most important issues is whether centrosome defects are a cause or consequence of the in-situ carcinoma phenotype. This is an issue of overriding importance in that the identification of the mechanism by which centrosome abnormalities arise may lead to both predictive testing and cancer-specific therapeutic interventions. There are many ways in which centrosome defects can arise. These include changes in proteins involved in cell cycle control, in centrosome structure or function and in DNA repair. For instance, mutation or elimination of p53 **(54-56)**, or p53 downstream effectors/regulators such as Mdm2 **(57)**, $p21^{Waf/Cip1}$ **(54, 57, 58)** and GADD45 **(44, 59)** induce centrosome abnormalities. Abrogation of postmitotic p53 dependent checkpoints may be critical in allowing tetraploid cells with supernumeray centrosomes to continue to cycle **(60-64)**. Similarly, alteration in the levels of centrosome-associated proteins such as pericentrin **(21, 27)**, γ-tubulin **(65)** aurora **(24, 25, 28)** polo **(66)**, TACC **(67)** and RanBP **(68)** lead to abnormal centrosomes. Moreover, mutation or functional abrogation of proteins involved in DNA repair such as Xrcc3 **(69)**, Xrcc2 **(69)**, BRCA1 **(70, 71)**, BRCA2 **(70, 72, 73)**, Mre11 **(74)**, DNA polymerase beta **(75)**, or genome damage signaling proteins such as ATR **(76)** can also lead to centrosome abnormalities. Lastly, centrosome abnormalities can arise by mutation of the adenomatous polyposis coli gene (APC) whose product interacts with microtubules **(77)**, by cytokinesis failure **(24)** and by ectopic assembly of centrosome components into acentriolar microtubule organizing centers **(9, 21, 27)**.

We do not know which of these mechanisms is responsible for inducing centrosome defects in carcinoma in situ or if another as yet unidentified mechanism/pathway is involved. We believe it is unlikely that p53 mutations can account for our findings. First p53 mutations are not common in DCIS **(78)** and PIN **(79)**. Second, centrosome defects in carcinoma in situ are not only numerical but also structural. Third, human somatic cells rendered p53-/- by targeted homologous recombination do not develop CIN or centrosome abnormalities unless challenged **(80)** Fourth, overexpression of endogenous p53, which correlates highly with mutated p53 occurred in less than 20% of CIC and PIN lesions (results not shown). Fifth, supernumerary centrosomes in p53-/- or p53 mutant cells **(54, 55)** may be secondary to the combined effects of cytokinesis failure and abrogation of postmitotic checkpoints thus allowing polyploid cells to re-enter the cell cycle and undergo mitosis with supernumerary centrosomes **(24)**. Under these conditions, cells with supernumerary centrosomes have the potential to perpetuate chromosome instability by missegregating chromosomes through multipolar mitoses.

Conclusion. In conclusion, we have shown that centrosome defects are present in a significant percentage of pre-invasive carcinomas and that they occur together with mitotic spindle defects and chromosome instability. We propose that centrosomes may contribute directly to chromosome missegregation and genetic instability and through this process, accelerate accumulation of genes with oncogenic mutations and loss of genes encoding tumor suppressors, as characteristically observed in human carcinoma.

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References.

1. Lengauer, C., Kinzler, K. W., and Vogelstein, B. Genetic instabilities in human cancers, Nature. *396:* 643-9., 1998.

2. Pihan, G. A., Purohit, A., Wallace, J., Knecht, H., Woda, B., Quesenberry, P., and Doxsey, S. J. Centrosome defects and genetic instability in malignant tumors, Cancer Res. *58:* 3974-85., 1998.

3. Cahill, D. P., Kinzler, K. W., Vogelstein, B., and Lengauer, C. Genetic instability and darwinian selection in tumours, Trends Cell Biol. *9:* M57-60., 1999.

4. Hahn, W. C. and Weinberg, R. A. Modelling the molecular circuitry of cancer, Nat Rev Cancer. *2:* 331-41., 2002.

5. Thiagalingam, S., Laken, S., Willson, J. K., Markowitz, S. D., Kinzler, K. W., Vogelstein, B., and Lengauer, C. Mechanisms underlying losses of heterozygosity in human colorectal cancers, Proc Natl Acad Sci U S A. *98:* 2698-702., 2001.

6. Lengauer, C., Kinzler, K. W., and Vogelstein, B. Genetic instability in colorectal cancers, Nature. *386:* 623-7., 1997.

7. Pihan, G. A. and Doxsey, S. J. The mitotic machinery as a source of genetic instability in cancer, Semin Cancer Biol. *9:* 289-302., 1999.

8. Brinkley, B. R. Managing the centrosome numbers game: from chaos to stability in cancer cell division, Trends Cell Biol. *11:* 18-21., 2001.

9. Doxsey, S. Re-evaluating centrosome function, Nat Rev Mol Cell Biol. *2:* 688-98., 2001.

10. Lingle, W. L. and Salisbury, J. L. The role of the centrosome in the development of malignant tumors, Curr Top Dev Biol. *49:* 313-29, 2000.

11. Marx, J. Cell biology. Do centrosome abnormalities lead to cancer?, Science. *292:* 426-9., 2001.

12. Winey, M. Cell cycle: driving the centrosome cycle, Curr Biol. *9:* R449-52., 1999.

13. Hinchcliffe, E. H. and Sluder, G. "It takes two to tango": understanding how centrosome duplication is regulated throughout the cell cycle, Genes Dev. *15:* 1167-81., 2001.

14. Khodjakov, A. and Rieder, C. L. Centrosomes enhance the fidelity of cytokinesis in vertebrates and are required for cell cycle progression, J Cell Biol. *153:* 237-42., 2001.

15. Piel, M., Nordberg, J., Euteneuer, U., and Bornens, M. Centrosome-dependent exit of cytokinesis in animal cells, Science. *291:* 1550-3., 2001.

16. Lingle, W. L., Lutz, W. H., Ingle, J. N., Maihle, N. J., and Salisbury, J. L. Centrosome hypertrophy in human breast tumors: implications for genomic stability and cell polarity, Proc Natl Acad Sci U S A. *95:* 2950-5., 1998.

17. Ghadimi, B. M., Sackett, D. L., Difilippantonio, M. J., Schrock, E., Neumann, T., Jauho, A., Auer, G., and Ried, T. Centrosome amplification and instability occurs exclusively in aneuploid, but not in diploid colorectal cancer cell lines, and correlates with numerical chromosomal aberrations, Genes Chromosomes Cancer. *27:* 183-90., 2000.

18. Haruki, N., Harano, T., Masuda, A., Kiyono, T., Takahashi, T., Tatematsu, Y., Shimizu, S., Mitsudomi, T., Konishi, H., Osada, H., and Fujii, Y. Persistent increase in chromosome instability in lung cancer: possible indirect involvement of p53 inactivation, Am J Pathol. *159:* 1345-52., 2001.

19. Kuo, K. K., Sato, N., Mizumoto, K., Maehara, N., Yonemasu, H., Ker, C. G., Sheen, P. C., and Tanaka, M. Centrosome abnormalities in human carcinomas of the gallbladder and intrahepatic and extrahepatic bile ducts, Hepatology. *31:* 59-64., 2000.

20. Lingle, W. L., Barrett, S. L., Negron, V. C., D'Assoro, A. B., Boeneman, K., Liu, W., Whitehead, C. M., Reynolds, C., and Salisbury, J. L. Centrosome amplification drives chromosomal instability in breast tumor development, Proc Natl Acad Sci U S A. *99:* 1978-83., 2002.

21. Pihan, G. A., Purohit, A., Wallace, J., Malhotra, R., Liotta, L., and Doxsey, S. J. Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression, Cancer Res. *61:* 2212-9., 2001.

22. Sato, N., Mizumoto, K., Nakamura, M., Nakamura, K., Kusumoto, M., Niiyama, H., Ogawa, T., and Tanaka, M. Centrosome abnormalities in pancreatic ductal carcinoma, Clin Cancer Res. *5:* 963-70., 1999.

23. Doxsey, S. The centrosome--a tiny organelle with big potential, Nat Genet. *20:* 104-6., 1998.

24. Meraldi, P., Honda, R., and Nigg, E. A. Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53-/- cells, Embo J. *21:* 483-92., 2002.

25. Bischoff, J. R., Anderson, L., Zhu, Y., Mossie, K., Ng, L., Souza, B., Schryver, B., Flanagan, P., Clairvoyant, F., Ginther, C., Chan, C. S., Novotny, M., Slamon, D. J., and Plowman, G. D. A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers, Embo J. *17:* 3052-65., 1998.

26. Gergely, F., Kidd, D., Jeffers, K., Wakefield, J. G., and Raff, J. W. D-TACC: a novel centrosomal protein required for normal spindle function in the early Drosophila embryo, Embo J. *19:* 241-52., 2000.

27. Purohit, A., Tynan, S. H., Vallee, R., and Doxsey, S. J. Direct interaction of pericentrin with cytoplasmic dynein light intermediate chain contributes to mitotic spindle organization, J Cell Biol. *147:* 481-92., 1999.

28. Zhou, H., Kuang, J., Zhong, L., Kuo, W. L., Gray, J. W., Sahin, A., Brinkley, B. R., and Sen, S. Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation, Nat Genet. *20:* 189-93., 1998.

29. Goepfert TM, A. Y., Zhong L, Medina D, Brinkley WR Centrosome amplification and overexpression of aurora A are early events in rat mammary carcinogenesis, Cancer Research. *62:* 4115-22, 2002.

30. Rosai, J. Akerman's Surgical Pathology, Eighth edition. New York: Mosby, 1996.

31. Lingle, W. L. and Salisbury, J. L. Altered centrosome structure is associated with abnormal mitoses in human breast tumors, Am J Pathol. *155:* 1941-51., 1999.

32. Saunders, W. S., Shuster, M., Huang, X., Gharaibeh, B., Enyenihi, A. H., Petersen, I., and Gollin, S. M. Chromosomal instability and cytoskeletal defects in oral cancer cells, Proc Natl Acad Sci U S A. *97:* 303-8., 2000.

33. Bulten, J., Poddighe, P. J., Robben, J. C., Gemmink, J. H., de Wilde, P. C., and Hanselaar, A. G. Interphase cytogenetic analysis of cervical intraepithelial neoplasia, Am J Pathol. *152:* 495-503., 1998.

34. Bright, R. K., Vocke, C. D., Emmert-Buck, M. R., Duray, P. H., Solomon, D., Fetsch, P., Rhim, J. S., Linehan, W. M., and Topalian, S. L. Generation and

genetic characterization of immortal human prostate epithelial cell lines derived from primary cancer specimens, Cancer Res. *57:* 995-1002., 1997.

35. Doxsey, S. J., Stein, P., Evans, L., Calarco, P. D., and Kirschner, M. Pericentrin, a highly conserved centrosome protein involved in microtubule organization, Cell. *76:* 639-50., 1994.

36. Crum, C. P. and McLachlin, C. M. Cervical intraepithelial neoplasia, J Cell Biochem Suppl. *23:* 71-9, 1995.

37. O'Connell, P., Pekkel, V., Fuqua, S., Osborne, C. K., and Allred, D. C. Molecular genetic studies of early breast cancer evolution, Breast Cancer Res Treat. *32:* 5-12, 1994.

38. Bostwick, D. G. Prostatic intraepithelial neoplasia is a risk factor for cancer, Semin Urol Oncol. *17:* 187-98., 1999.

39. Douglas-Jones, A. G., Gupta, S. K., Attanoos, R. L., Morgan, J. M., and Mansel, R. E. A critical appraisal of six modern classifications of ductal carcinoma in situ of the breast (DCIS): correlation with grade of associated invasive carcinoma, Histopathology. *29:* 397-409., 1996.

40. Schultz, L. B. and Weber, B. L. Recent advances in breast cancer biology, Curr Opin Oncol. *11:* 429-34., 1999.

41. Wolf, J. K. and Ramirez, P. T. The molecular biology of cervical cancer, Cancer Invest. *19:* 621-9, 2001.

42. Levine, D. S., Sanchez, C. A., Rabinovitch, P. S., and Reid, B. J. Formation of the tetraploid intermediate is associated with the development of cells with more than four centrioles in the elastase- simian virus 40 tumor antigen transgenic mouse model of pancreatic cancer, Proc Natl Acad Sci U S A. *88:* 6427-31., 1991.

43. Li, R., Yerganian, G., Duesberg, P., Kraemer, A., Willer, A., Rausch, C., and Hehlmann, R. Aneuploidy correlated 100% with chemical transformation of Chinese hamster cells, Proc Natl Acad Sci U S A. *94:* 14506-11., 1997.

44. Wang, X. W., Zhan, Q., Coursen, J. D., Khan, M. A., Kontny, H. U., Yu, L., Hollander, M. C., O'Connor, P. M., Fornace, A. J., Jr., and Harris, C. C. GADD45 induction of a G2/M cell cycle checkpoint, Proc Natl Acad Sci U S A. *96:* 3706-11., 1999.

45. Weinberg, D. S. and Weidner, N. Concordance of DNA content between prostatic intraepithelial neoplasia and concomitant invasive carcinoma. Evidence that prostatic intraepithelial neoplasia is a precursor of invasive prostatic carcinoma, Arch Pathol Lab Med. *117:* 1132-7., 1993.

46. Bornens, M. Centrosome composition and microtubule anchoring mechanisms, Curr Opin Cell Biol. *14:* 25-34., 2002.

47. Niu, M. Y., Mills, J. C., and Nachmias, V. T. Development of polarity in human erythroleukemia cells: roles of membrane ruffling and the centrosome, Cell Motil Cytoskeleton. *36:* 203-15, 1997.

48. Yvon, A. M., Walker, J. W., Danowski, B., Fagerstrom, C., Khodjakov, A., and Wadsworth, P. Centrosome reorientation in wound-edge cells is cell type specific, Mol Biol Cell. *13:* 1871-80., 2002.

49. van de Vijver, M. J. Ductal carcinoma in situ of the breast: histological classification and genetic alterations, Recent Results Cancer Res. *152:* 123-34, 1998.

50. von Eschenbach, A. C. The challenge of prostate cancer, CA Cancer J Clin. *49:* 262-3., 1999.

51. Munger, K. The role of human papillomaviruses in human cancers, Front Biosci. *7:* d641-9., 2002.

52. Duensing, S. and Munger, K. Centrosome abnormalities, genomic instability and carcinogenic progression, Biochim Biophys Acta. *2:* M81-8, 2001.

53. Ring, D., Hubble, R., and Kirschner, M. Mitosis in a cell with multiple centrioles, J Cell Biol. *94:* 549-56., 1982.

54. Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S., and Vande Woude, G. F. Abnormal centrosome amplification in the absence of p53, Science. *271:* 1744-7., 1996.

55. Tarapore, P., Horn, H. F., Tokuyama, Y., and Fukasawa, K. Direct regulation of the centrosome duplication cycle by the p53- p21Waf1/Cip1 pathway, Oncogene. *20:* 3173-84., 2001.

56. Wang, X. J., Greenhalgh, D. A., Jiang, A., He, D., Zhong, L., Medina, D., Brinkley, B. R., and Roop, D. R. Expression of a p53 mutant in the epidermis of transgenic mice accelerates chemical carcinogenesis, Oncogene. *17:* 35-45., 1998.

57. Carroll, P. E., Okuda, M., Horn, H. F., Biddinger, P., Stambrook, P. J., Gleich, L. L., Li, Y. Q., Tarapore, P., and Fukasawa, K. Centrosome hyperamplification in human cancer: chromosome instability induced by p53 mutation and/or Mdm2 overexpression, Oncogene. *18:* 1935-44., 1999.

58. Mantel, C., Braun, S. E., Reid, S., Henegariu, O., Liu, L., Hangoc, G., and Broxmeyer, H. E. p21(cip-1/waf-1) deficiency causes deformed nuclear architecture, centriole overduplication, polyploidy, and relaxed microtubule damage checkpoints in human hematopoietic cells, Blood. *93:* 1390-8., 1999.

59. Hollander, M. C., Sheikh, M. S., Bulavin, D. V., Lundgren, K., Augeri-Henmueller, L., Shehee, R., Molinaro, T. A., Kim, K. E., Tolosa, E., Ashwell, J. D., Rosenberg, M. P., Zhan, Q., Fernandez-Salguero, P. M., Morgan, W. F., Deng, C. X., and Fornace, A. J., Jr. Genomic instability in Gadd45a-deficient mice, Nat Genet. *23:* 176-84., 1999.

60. Andreassen, P. R., Lohez, O. D., Lacroix, F. B., and Margolis, R. L. Tetraploid state induces p53-dependent arrest of nontransformed mammalian cells in G1, Mol Biol Cell. *12:* 1315-28., 2001.

61. Casenghi, M., Mangiacasale, R., Tuynder, M., Caillet-Fauquet, P., Elhajouji, A., Lavia, P., Mousset, S., Kirsch-Volders, M., and Cundari, E. p53-independent apoptosis and p53-dependent block of DNA rereplication following mitotic spindle inhibition in human cells, Exp Cell Res. *250:* 339-50., 1999.

62. Khan, S. H. and Wahl, G. M. p53 and pRb prevent rereplication in response to microtubule inhibitors by mediating a reversible G1 arrest, Cancer Res. *58:* 396-401., 1998.

63. Lanni, J. S. and Jacks, T. Characterization of the p53-dependent postmitotic checkpoint following spindle disruption, Mol Cell Biol. *18:* 1055-64., 1998.

64. Minn, A. J., Boise, L. H., and Thompson, C. B. Expression of Bcl-xL and loss of p53 can cooperate to overcome a cell cycle checkpoint induced by mitotic spindle damage, Genes Dev. *10:* 2621-31., 1996.

65. Shu, H. B. and Joshi, H. C. Gamma-tubulin can both nucleate microtubule assembly and self-assemble into novel tubular structures in mammalian cells, J Cell Biol. *130:* 1137-47., 1995.

66. Conn, C. W., Hennigan, R. F., Dai, W., Sanchez, Y., and Stambrook, P. J. Incomplete cytokinesis and induction of apoptosis by overexpression of the mammalian polo-like kinase, Plk3, Cancer Res. *60:* 6826-31., 2000.

67. Raff, J. W. and Glover, D. M. Centrosomes, and not nuclei, initiate pole cell formation in Drosophila embryos, Cell. *57:* 611-9., 1989.

68. Wiese, C., Wilde, A., Moore, M. S., Adam, S. A., Merdes, A., and Zheng, Y. Role of importin-beta in coupling Ran to downstream targets in microtubule assembly, Science. *291:* 653-6., 2001.

69. Griffin, C. S., Simpson, P. J., Wilson, C. R., and Thacker, J. Mammalian recombination-repair genes XRCC2 and XRCC3 promote correct chromosome segregation, Nat Cell Biol. *2:* 757-61., 2000.

70. Bertwistle, D. and Ashworth, A. The pathology of familial breast cancer: How do the functions of BRCA1 and BRCA2 relate to breast tumour pathology?, Breast Cancer Res. *1:* 41-7, 1999.

71. Xu, X., Weaver, Z., Linke, S. P., Li, C., Gotay, J., Wang, X. W., Harris, C. C., Ried, T., and Deng, C. X. Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells, Mol Cell. *3:* 389-95., 1999.

72. Kraakman-van der Zwet, M., Overkamp, W. J., van Lange, R. E., Essers, J., van Duijn-Goedhart, A., Wiggers, I., Swaminathan, S., van Buul, P. P., Errami, A., Tan, R. T., Jaspers, N. G., Sharan, S. K., Kanaar, R., and Zdzienicka, M. Z. Brca2 (XRCC11) deficiency results in radioresistant DNA synthesis and a higher frequency of spontaneous deletions, Mol Cell Biol. *22:* 669-79., 2002.

73. Tutt, A., Gabriel, A., Bertwistle, D., Connor, F., Paterson, H., Peacock, J., Ross, G., and Ashworth, A. Absence of Brca2 causes genome instability by chromosome breakage and loss associated with centrosome amplification, Curr Biol. *9:* 1107-10., 1999.

74. Yamaguchi-Iwai, Y., Sonoda, E., Sasaki, M. S., Morrison, C., Haraguchi, T., Hiraoka, Y., Yamashita, Y. M., Yagi, T., Takata, M., Price, C., Kakazu, N., and Takeda, S. Mre11 is essential for the maintenance of chromosomal DNA in vertebrate cells, Embo J. *18:* 6619-29., 1999.

75. Bergoglio, V., Pillaire, M. J., Lacroix-Triki, M., Raynaud-Messina, B., Canitrot, Y., Bieth, A., Gares, M., Wright, M., Delsol, G., Loeb, L. A., Cazaux, C., and Hoffmann, J. S. Deregulated DNA polymerase beta induces chromosome instability and tumorigenesis, Cancer Res. *62:* 3511-4., 2002.

76. Smith, L., Liu, S. J., Goodrich, L., Jacobson, D., Degnin, C., Bentley, N., Carr, A., Flaggs, G., Keegan, K., Hoekstra, M., and Thayer, M. J. Duplication of ATR inhibits MyoD, induces aneuploidy and eliminates radiation-induced G1 arrest, Nat Genet. *19:* 39-46., 1998.

77. Fodde, R., Kuipers, J., Rosenberg, C., Smits, R., Kielman, M., Gaspar, C., van Es, J. H., Breukel, C., Wiegant, J., Giles, R. H., and Clevers, H. Mutations in the APC tumour suppressor gene cause chromosomal instability, Nat Cell Biol. *3:* 433-8., 2001.

78. Rajan, P. B., Scott, D. J., Perry, R. H., and Griffith, C. D. p53 protein expression in ductal carcinoma in situ (DCIS) of the breast, Breast Cancer Res Treat. *42:* 283-90., 1997.

79. Takayama, H., Shin, M., Nonomura, N., Okuyama, A., and Aozasa, K. p53 mutations in prostatic intraepithelial neoplasia and concurrent carcinoma: analysis of laser capture microdissected specimens from non- transition and transition zones, Jpn J Cancer Res. *91:* 941-7., 2000.

80. Bunz, F., Fauth, C., Speicher, M. R., Dutriaux, A., Sedivy, J. M., Kinzler, K. W., Vogelstein, B., and Lengauer, C. Targeted inactivation of p53 in human cells does not result in aneuploidy, Cancer Res. *62:* 1129-33., 2002.

Figure Legends.

Fig 1. Centrosome defects occur in carcinoma in situ. Photomicrographs of normal epithelium (A, C, and E) and adjacent in situ carcinoma (B, D and F) immunostained with antibodies to pericentrin to visualize centrosomes. Magnifications are all the same (1000X). In normal epithelia, centrosomes are round and uniform in size (arrowheads, A, C and E) while in carcinoma in situ they are larger (arrowheads in B, D, F), multiple (B) or structurally abnormal (arrowheads in D and F). Nuclei are stained light blue with hematoxylin. Inset in D shows higher magnification of an elongated centrosome. Note that nucleus and cell in B are considerably larger than those in adjacent normal epithelial cells (A).

Fig 2. Centrosome defects are prevalent in carcinoma in situ. Centrosome defects are present in 62, 75 and 28 percent of CIC (A), DCIS (B) and PIN (C) lesions, respectively (N, normal epithelia). First column (A-C), cumulative defects; second column (A'-C'), breakdown of centrosome defects by category (#, number, Sz, size, Sh, shape).

Fig 3. The incidence of centrosome defects increases with increasing histologic grade. The cumulative incidence of centrosome defects in each pre-invasive lesion (left column) includes grades 1-3 for CIC (A, 1-3) and low (L) and high (H) grades for DCIS (E) and PIN (I). N, normal epithelium. Each subcategory of centrosome defects increases

with grade including increased centrosome number (B, F, J), shape abnormalities (C, G, K) and size (D, H, L).

Fig 4. Mitotic spindle defects are common in CIC and DCIS. Examples of bipolar mitotic spindles immunostained with γ-tubulin in CIC and DCIS (A and C, respectively). Examples of multipolar spindles (B, CIC, D, F, DCIS) and multiple spindles (E, DCIS). Quantitative analysis of the number of bipolar spindles (x axis) and multipolar spindles (y axis) in each CIC lesion (G) and DCIS lesion (H). Each circle represents a single lesion. Filled circles represent lesions with ten or more mitoses and were included in the estimation of the extent of mitotic spindle defects in CIC and DCIS. On average 10% and 17% of the spindles, in CIC and DCIS lesions with more than 10 immunostained spindles (red circles in G and H) are abnormal.

Fig 5. Centrosome abnormalities correlate with chromosome instability in carcinoma in-situ. Examples of in situ hybridization reactions performed on samples of CIC (B), DCIS (D) and PIN (F). Many cells have more than two signals for chromosome #8 (arrowheads in B, D, F) and thus exhibit chromosome instability (CIN+). Cells in adjacent normal epithelium (A, C, E) rarely have more than two signals. Quantitative analysis of chromosomal instability (CIN+) in CIC (G), DCIS (H) and PIN (I) lesions with normal centrosomes (N) or abnormal centrosomes (A). CIN is present in most lesions with abnormal centrosomes and a small fraction of lesions lacking centrosome abnormalities.

Figure 1

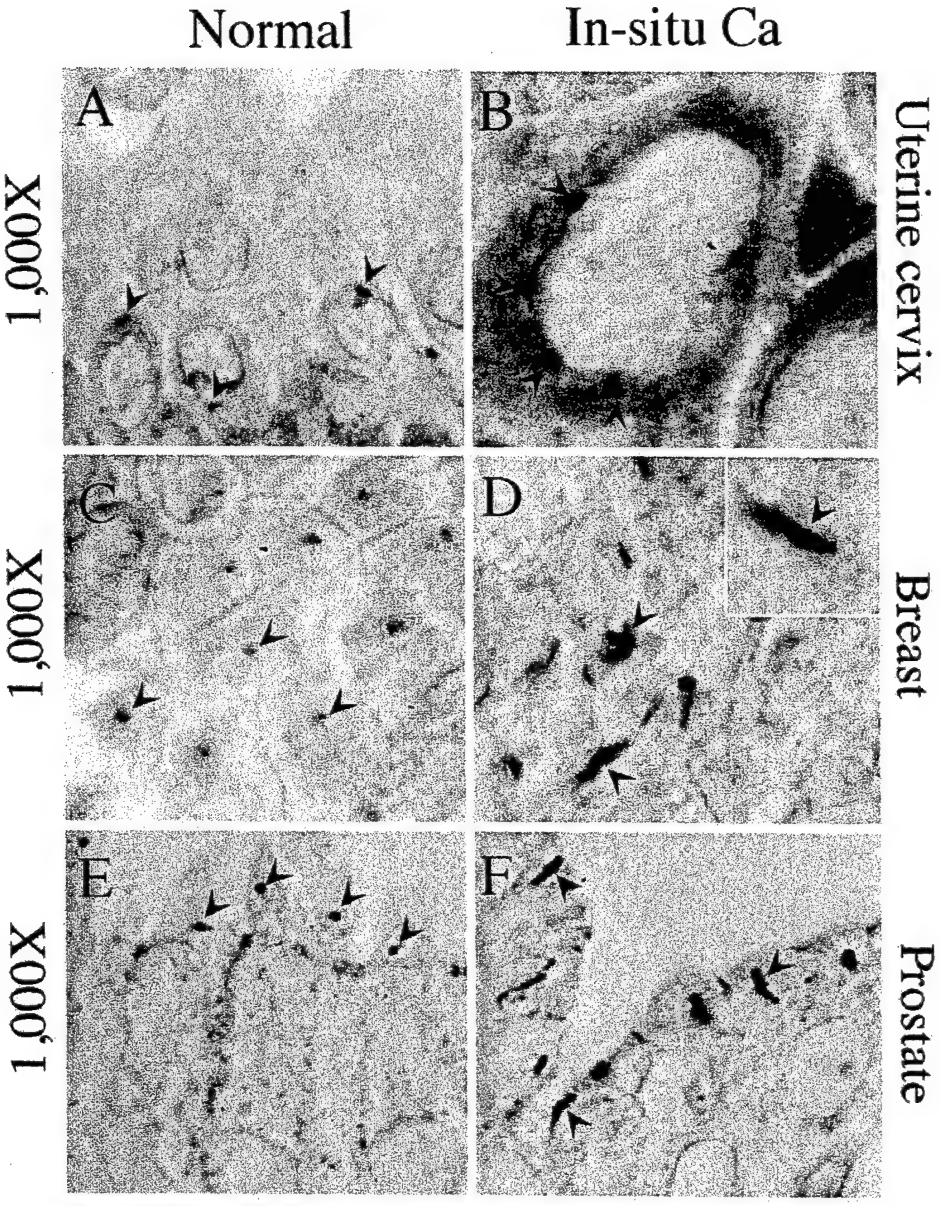

Normal
In-situ Ca
A
B
C
D
E
F
1,000X
1,000X
1,000X
Uterine cervix
Breast
Prostate

Figure 2

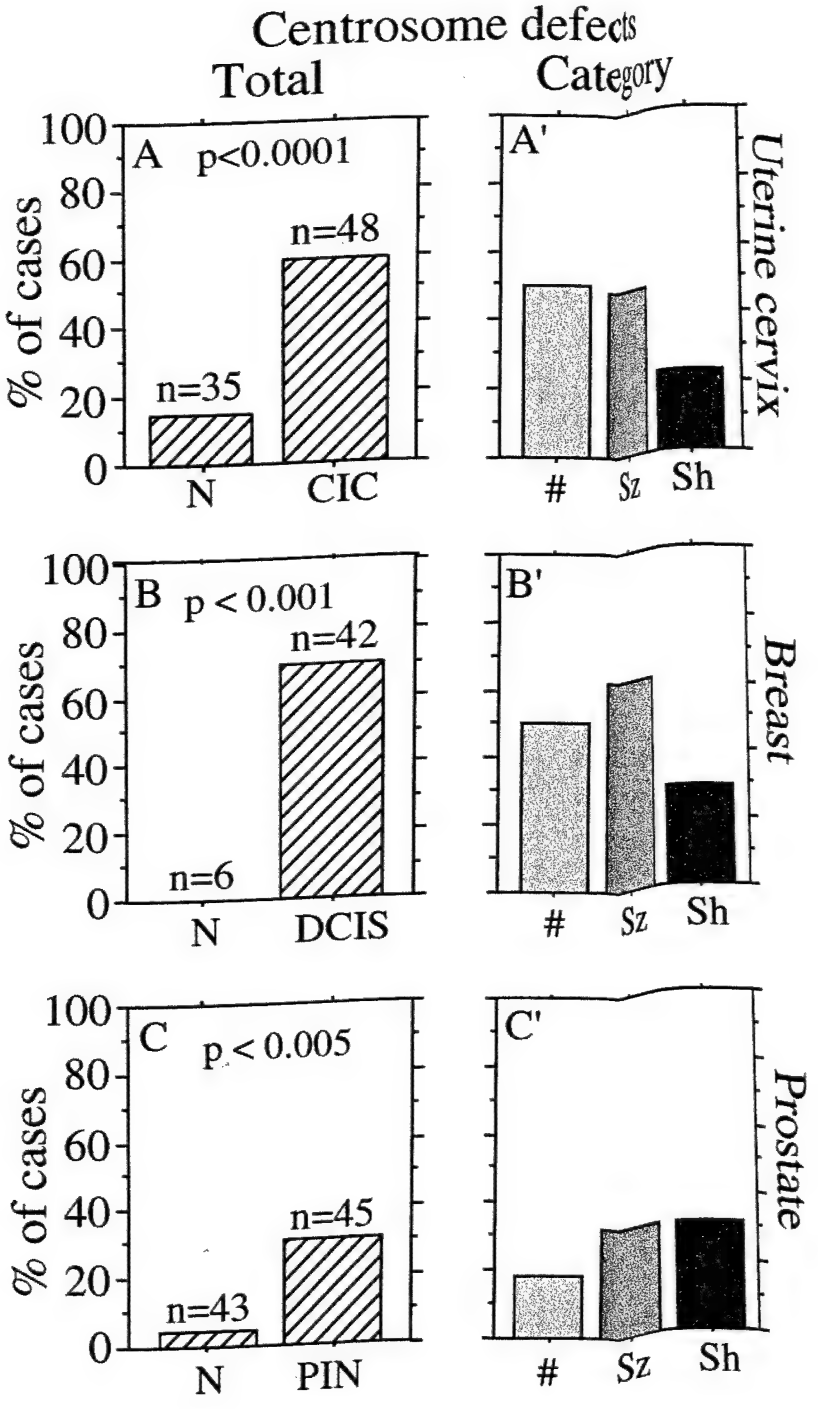

Centrosome defects
Total
Category
A
p<0.0001
n=48
n=35
% of cases
100
80
60
40
20
0
N
CIC
A'
Uterine cervix
#
Sz
Sh
B
p < 0.001
n=42
n=6
DCIS
B'
Breast
C
p < 0.005
n=45
n=43
PIN
C'
Prostate

Figure 3

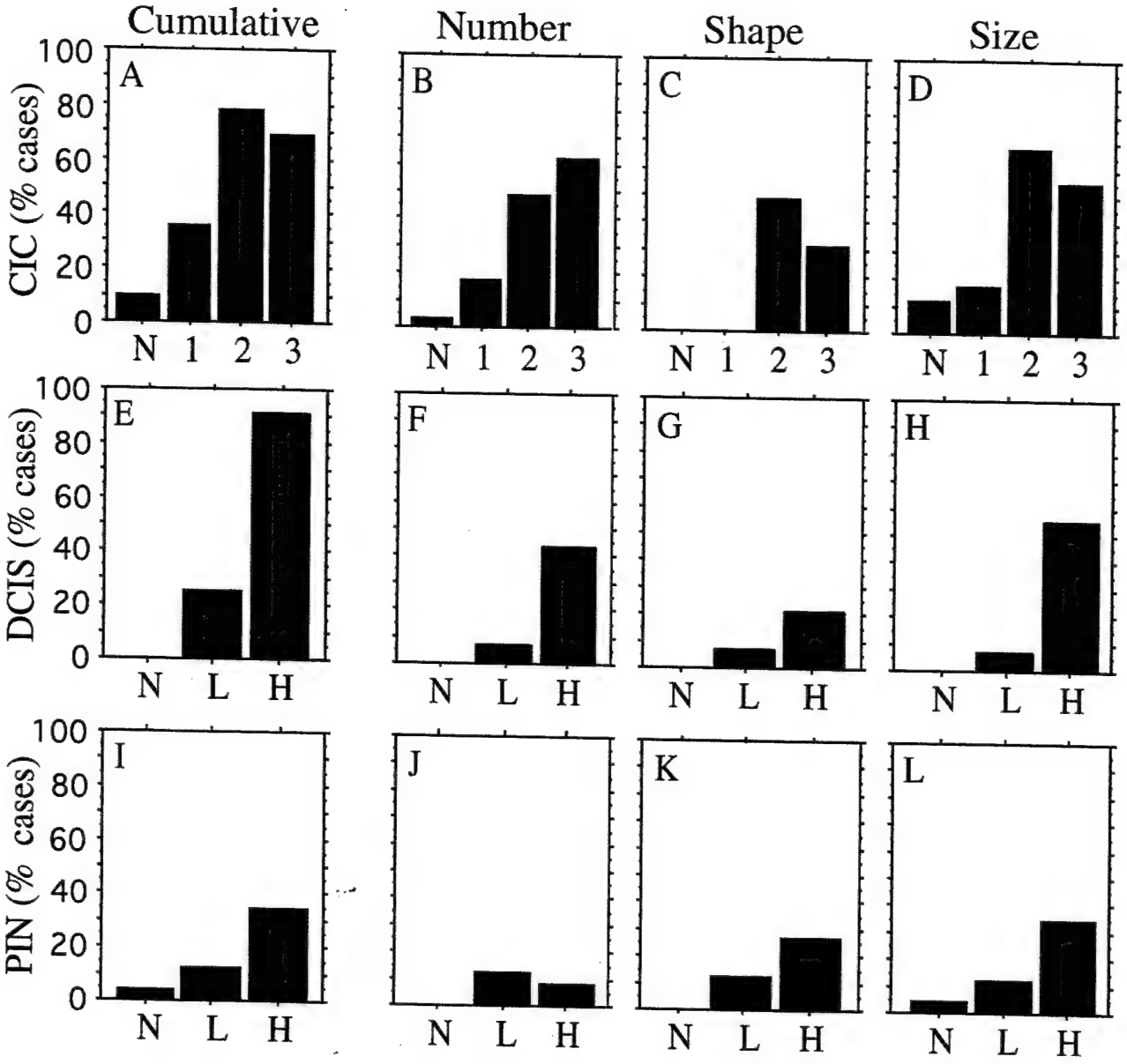

Cumulative
Number
Shape
Size
CIC (% cases)
DCIS (% cases)
PIN (% cases)
100
80
60
40
20
0
A
B
C
D
E
F
G
H
I
J
K
L
N 1 2 3
N L H

Figure 4

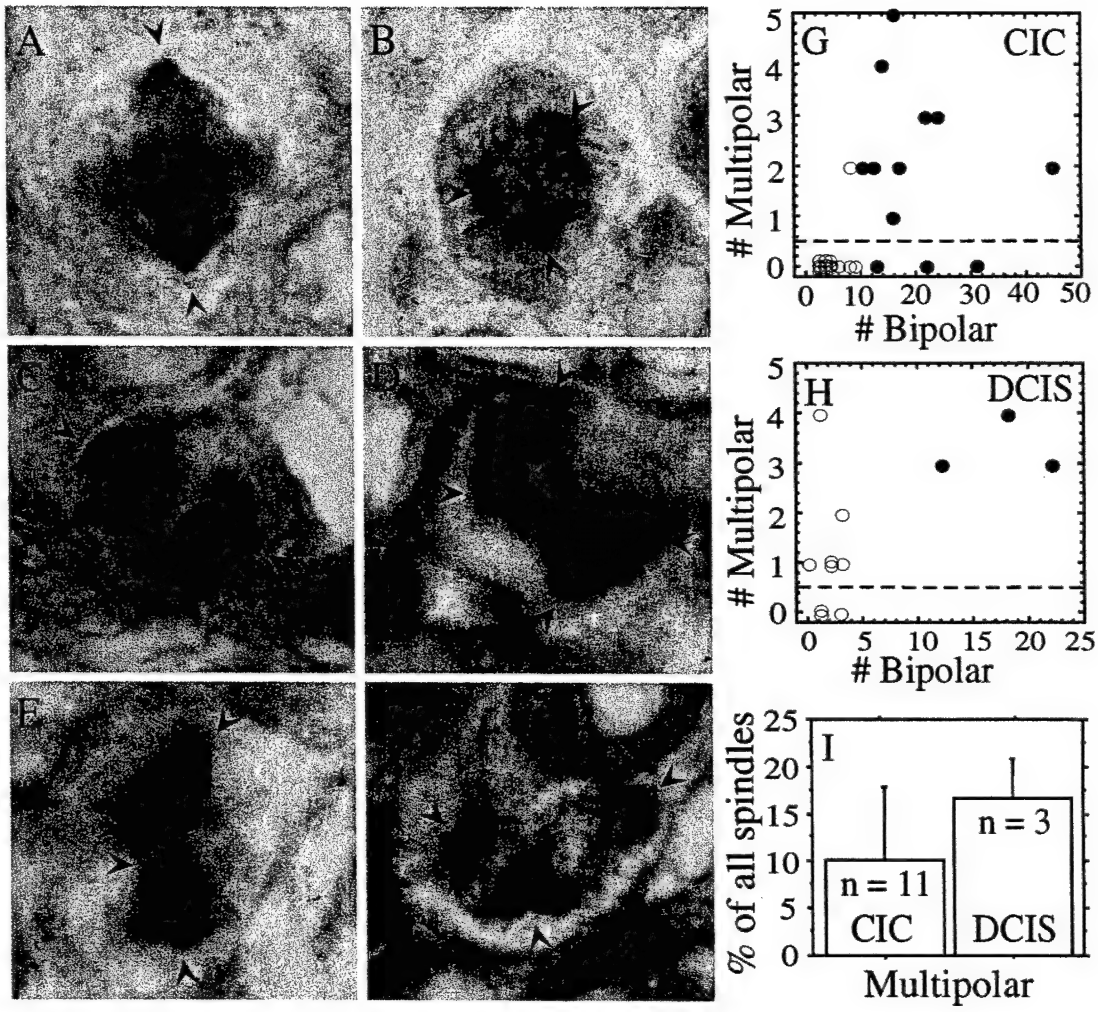
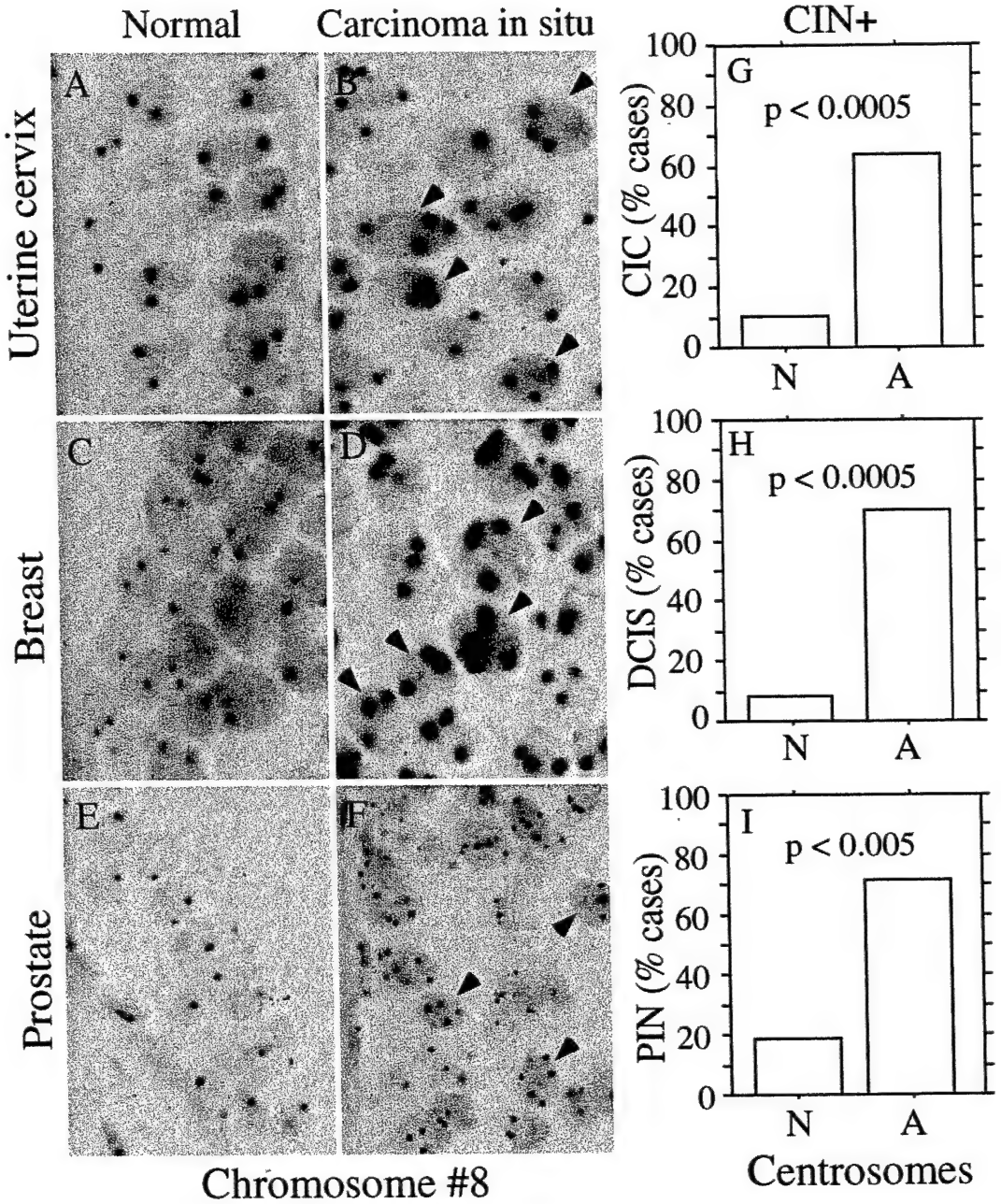

A
B
C
D
E
G
CIC
Multipolar
Bipolar
H
DCIS
Multipolar
Bipolar
I
% of all spindles
n = 11
CIC
n = 3
DCIS
Multipolar

Figure 5


Normal
Carcinoma in situ
Uterine cervix
Breast
Prostate
A
B
C
D
E
F
Chromosome #8
CIN+
G
p < 0.0005
CIC (% cases)
H
p < 0.0005
DCIS (% cases)
I
p < 0.005
PIN (% cases)
100
80
60
40
20
0
N
A
Centrosomes

A Novel Human Protein of the Maternal Centriole is Required for the Final Stages of Cytokinesis and Entry into S-phase.

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Centrosomes nucleate microtubules and contribute to mitotic spindle function. They also participate in cytokinesis and cell cycle progression in ways that are incompletely understood. Here we describe a novel human protein called centriolin that localizes to the maternal centriole and functions in both processes. Centriolin silencing induces cytokinesis failure by a novel mechanism whereby cells remain interconnected by long intercellular bridges and fail to cleave. Cells continue to cycle, re-enter mitosis and form multicelluar syncytia. They eventually divide or undergo apoptosis specifically during the protracted cytokinesis period. Ultimately, viable cells arrest in G1/G0. The cytokinesis activity lies in a centriolin domain homologous to proteins of yeast regulatory pathways that control late stages of mitosis, and this domain binds Bub2p, another component of the yeast pathway. We conclude that centriolin is required for a late stage of vertebrate cytokinesis perhaps during the final cell cleavage event, and plays a role in progression into S-phase.

Introduction

Centrosomes are the major microtubule nucleating organelles in most vertebrate cells (see [1]). In mitosis they contribute to spindle organization and function and in interphase they organize microtubule arrays that serve as tracks for transporting proteins, organelles and chromosomes. The centrosome also anchors regulatory molecules and may serve as a central site that receives, integrates and transmits signals that regulate fundamental cellular functions. The core of the centrosome is comprised of a pair of centrioles, microtubule barrels that appear to anchor microtubules [2,3]. Each centriole is surrounded by pericentriolar material or centrosome matrix, which nucleates the growth of new microtubules and seems to be organized by the centrioles [4]. Although best known for their role in microtubule nucleation, recent data suggests that centrosomes also play key roles in cytokinesis and cell cycle progression.

A role for centrosomes in defining the site of cell cleavage during cytokinesis has been suggested for some time [5]. Recent studies with vertebrate cells provide evidence for a direct link between centrosome activity and completion of cytokinesis. Elimination of

centrosomes from interphase cells by removal with a microneedle [6] or from mitotic cells by laser ablation [7], caused cytokinesis defects, arrest or failure. In another study, it was shown that during the final stages of cytokinesis the maternal centriole moved to the intercellular bridge, the microtubule-filled interconnection between nascent daughter cells [8]. This centriole re-positioning correlated with bridge narrowing and microtubule depolymerization, and centriole movement away from the bridge correlated with cell cleavage or abscission. The authors suggested that the maternal centriole may anchor a regulatory pathway that controls the final stages of cell division in vertebrate cells. This would be analogous to regulatory pathways anchored at spindle pole bodies (the centrosome equivalent) in budding and fission yeasts that control mitotic exit and cytokinesis (for review see [9-11]). However, no vertebrate pathway analogous to the mitotic exit network (MEN) in budding yeast or septation initiation network (SIN) in fission yeast has been identified [12,13]. Moreover, the role of centrosome-associated molecules in the process of cytokinesis is poorly understood.

In addition to their role in cytokinesis, centrosomes may be involved in cell cycle progression. When centrosomes were removed from vertebrate cells, half of the cells completed mitosis but did not initiate DNA replication [6,7]. The authors suggested that centrosomes controlled entry into S-phase by recruiting or concentrating "core" centrosome molecules required for this process or that they indirectly activated a cellular checkpoint that monitored aberrant centrosome number. In another experimental system, vertebrate cells treated with cytochalasin D to inhibit actin-mediated cell cleavage also arrested cells in G1 as binucleate cells with supernumerary centrosomes [14]. While these results suggest that changes in centrosome number can affect entry into S-phase, the precise role of centrosomes in cell cycle progression in vertebrate cells will require identification of the molecular components and pathways that control these events.

In this manuscript we identify a novel component of the vertebrate maternal centriole called centriolin. Abrogation of centriolin function by siRNA silencing, overexpression or antibody inhibition produces cytokinesis failure and G1 arrest, just as seen when centrosomes are experimentally eliminated from cells. Centriolin silencing produces a

novel cytokinesis phenotype in which dividing cells remain interconnected by long strands of cytoplasm and fail to cleave. The cytokinesis activity lies in a centriolin domain that is homologous to the MEN/SIN components Nud1p/Cdc11p and binds the Nud1p-interacting GTPase-activating protein Bub2p. We conclude that centriolin is required for a distinct step in the final stages of vertebrate cytokinesis and can influence entry into S-phase.

Results

Identification and cloning of a novel protein localized to the maternal centriole and intercellular bridge.

Using sera from patients with the autoimmune disease scleroderma that react with centrosomes [15] we screened a human placenta λgt11 cDNA expression library to identify genes encoding the autoantigens. Of the 3 x 10^6 clones screened, only one of 1.7 Kb was identified indicating that the mRNA for this molecule was rare. The full-length cDNA was obtained and the protein encoded by the cDNA was called centriolin (see below and Methods). The amino acid sequence of centriolin predicted a protein with several coiled-coil regions interrupted by noncoiled domains [16] (Fig. 1a). Two domains within the centriolin sequence shared homology with human oncogenic transforming acidic coiled coil proteins (TACCs) which localize to centrosomes and are implicated in microtubule stabilization and spindle function [17]. Another domain of centriolin was homologous to human stathmin, an oncogenic protein involved in microtubule destabilization [18]. The carboxyl terminus of centriolin was identical to CEP110, a naturally occurring fusion to the fibroblast growth factor (FGF) receptor that localizes to centrosomes, is oncogenic and is of unknown function [19]. A region of centriolin near the amino terminus shared homology with Nud1p and Cdc11p, budding and fission yeast spindle pole body proteins that anchor components of the yeast MEN and SIN, respectively, and are required for completion of mitosis and cytokinesis (see [9-11,13]). The 120 amino acid region of shared homology between the Nud1 domain, Nud1p and CDc11p all have leucine rich repeats and are predicted to form β helix structures in tertiary structure prediction programs.

Antibodies raised against recombinant centriolin recognized a band of ~270 kD on Western blots of isolated centrosome fractions while preimmune sera showed no specific bands (Fig. 1b, panels 3 and 4). The protein was not detected by Western blotting of whole-cell lysates, consistent with the probable low abundance of this and other centrosome autoantigens [15]. In vitro translation and overexpression of the protein in mammalian cells using the full-length cDNA produced a protein with a molecular weight similar to the endogenous protein (Fig. 1b, panels 1 and 2).

Immunofluorescence microscopy demonstrated that centriolin was localized to centrosomes in a wide variety of species including human, monkey, hamster, mouse, and Xenopus (Fig. 1, 2, data not shown). Centrosome localization was confirmed by showing that a hemagglutinin (HA)-tagged centriolin protein ectopically expressed in COS cells localized to centrosomes (Fig. 2a). The endogenous protein was present on the centrosome throughout the cell cycle. In late G1/early S phase centrosomes begin to duplicate and by G2/M duplication is usually completed. During the duplication process, centriolin was present on only one of the two duplicating centrosomes although other proteins such as γ tubulin were found on both (Fig. 1c, G2 cell). Beginning at late G2/prometaphase, dim staining was observed next to a brightly-stained centrosome. By metaphase when centrosomes become "mature", both centrosomes had equally high levels of centriolin and were higher than any other cell cycle stage. This demonstrates that centriolin is a marker for centrosome maturation, a characteristic shared with cenexin [20] and ninein [21]. At the metaphase to anaphase transition, centriolin staining diminished at centrosomes and reached its lowest levels by late anaphase/telophase. During cytokinesis, centriolin sometimes appeared as one or two dots adjacent to the intercellular bridge suggesting that the centrosome/centriole had moved to this site (Fig. 1c, Telo early, middle panels). This staining pattern was consistent with recent time lapse imaging experiments showing that the maternal centriole translocates to the intercellular bridge during cytokinesis [8]. Centriolin next appeared as diffusely organized material within the intercellular bridge and ultimately became concentrated at the midbody (Fig. 1c, Telo late, bottom panels).

The organization of centriolin at the centrosome was more precisely determined by serum-starving cells to induce growth of a primary cilium from the maternal centriole [22]. In these cells, centriolin staining was confined to the maternal centriole underlying the cilium (Fig. 2b). Immunogold electron microscopy on centrosome fractions [15,23] confirmed localization to the maternal centriole (Fig. 2e, "M") and further demonstrated that the protein was concentrated on subdistal appendages, specialized substructures of the maternal centriole implicated in microtubule anchoring (Fig. 2c-e) [2,3]. Based on its

centriolar localization the protein was named centriolin. Centriolin was also found at noncentrosomal apical bands of material in specialized epithelial cells that lack proteins involved in microtubule nucleation and appear to anchor the minus ends of microtubules [24] (Fig. 2 f,g).

Centriolin silencing by siRNA induces cytokinesis failure and a novel cytokinesis phenotype.

To determine the function of centriolin, we reduced its levels using small interfering RNAs (siRNAs) [25]. Treatment of telomerase-immortalized diploid human retinal pigment epithelial cells (RPE-1) [26] with centriolin-specific siRNAs caused a significant reduction in centriolin mRNA levels (Fig. 3a). Although we were unable to examine protein levels by Western blotting of whole cell lysates due to the rare nature of this and other centrosome autoantigens [15], immunofluorescence staining demonstrated that centriolin was undetectable or greatly reduced at centrosomes in most cells (86%, n=1012). Quantitative analysis showed that immunofluorescence signals at individual centrosomes was significantly below those in cells treated with control lamin A/C siRNA despite severe disruption of the nuclear lamina in the latter (Fig. 3b) [25]. The midbody staining of centriolin was also reduced in cells treated with siRNAs targeting centriolin (data not shown).

Because centriolin shares homology with proteins known to affect microtubule organization and cytokinesis, we examined cells with reduced centriolin for defects in these functions. The most obvious cellular change detected in RPE-1 cells with reduced centriolin was a dramatic increase in the percentage of late-stage mitotic cells (Fig. 3c, ~70-fold increase, 35% versus 0.5% in controls). In addition, we observed an increase in the percentage of binucleate cells in three different cell lines demonstrating that a certain proportion of cells failed to cleave (Fig. 3d). The incidence of binucleate cells was significantly greater than controls although somewhat lower than that observed for some other proteins involved in cytokinesis [27-29]. A similar cytokinesis phenotype was observed with a second set of siRNAs targeting a different centriolin sequence and with morpholino antisense DNA oligonucleotides targeting centriolin (data not shown).

The dramatically high percentage of cells in late mitotic stages suggested a unique cytokinesis defect in these cells. When carefully analyzed by immunofluorescence microscopy, cells with reduced centriolin appeared to be arrested or delayed in the final stages of cytokinesis. Most cells retained intercellular bridges of varying length and thickness (arrowheads, Fig. 3m,n). In some cases, cells remained connected even though one or both of the future daughter cells had re-entered mitosis ("M", Fig. 3n). Some cells failed to cleave forming syncytia with two, three of four cells remaining interconnected (Fig. 3m,n). Although midbodies appeared normal by microtubule labeling and γ tubulin labeling during the early stages of cytokinesis (telophase, Fig. 3j, data not shown), they were not detected in cells with long persistent intercellular bridges.

A more complete understanding of the mechanism of cytokinesis failure was obtained by imaging living HeLa cells treated with centriolin-specific siRNAs (Fig. 4). As expected, control cells (lamin siRNA) performed a distinct cell cleavage event with normal timing (average 2 hours after mitosis) and immediately flattened and crawled apart (Fig. 4a). Cells silenced for centriolin progressed normally through mitosis (Fig. 4e, Fig. 3g-j) and sometimes cleaved normally, but most failed to cleave or cleaved after prolonged periods of time (up to 23.2 hours after metaphase, Fig. 4b-d, f). These cells arrested in a unique post-telophase state. Most were unusually elongated, each with a persistent intercellular bridge of variable diameter. The intercellular bridges were very dynamic. They alternated between thin threads of interconnecting cytoplasm to very thick interconnections of large diameter that appeared able to produce membrane ruffles (Fig. 4b, 5:50, arrow). Midbodies were not detected within persistent interconnections between cells suggesting that they were lost sometime during the protracted period spent in cytokinesis. Interconnected cells sometimes coalesced to form single cells then quickly moved apart again (Fig. 4d). They sometimes made multiple failed attempts at cleavage, but in no case did we observe a cell that formed a stable binucleate. Cells that retained intercellular connections for long periods of time continued to progress through the cell cycle. To our surprise, some cells re-entered the next mitosis while still interconnected and produced interconnected "progeny" that formed two to four-cell syncytia, thus confirming our

results from indirect immunofluorescence on RPE-1 cells. In some cases, cells that remained interconnected for long periods of time appeared to undergo apoptosis. They showed extensive blebbing, increased phase-density, decreased size and lifted from the substrate (Fig. 4b, upper cell, 7:20).

We next examined microtubule organization in cells with reduced centriolin. At all cell cycle stages, microtubule organization appeared normal including the spindle midzone microtubules in anaphase and midbody microtubules in telophase (Fig. 3e-j). Microtubule nucleation from centrosomes also appeared normal (Fig. 3k,l) although a slight delay was sometimes observed within the first minute or two. γ tubulin, a marker for centrosome-associated microtubule nucleation, was localized normally to centrosomes (Fig. 3b) as were other several other centrosome antigens including GCP-2 [30] and cNap-1 [31] (data not shown). These data indicate that cytokinesis failure did not result from disruption of microtubules or centrosomes.

Overexpression of the centriolin Nud1 domain induces cytokinesis failure in a microtubule-independent manner.

We next tested the effect of ectopic expression of centriolin and its Nud1 domain on cytokinesis and microtubule organization (Fig. 5a-g). The most striking defect in COS-7 cells expressing HA-tagged centriolin was a high percentage of binucleate cells that comprised over 20% of the population (Fig. 5g). In addition, we observed an increase in the proportion of cells in telophase and cells with cytokinesis defects. The most common defect was the presence of microtubule bundles within intercellular bridges in late stage telophase cells (Fig. 5a). This suggested that microtubule bundling may contribute to the observed cytokinesis failure (binucleate formation). However, this was not the case. We found that overexpression of the 120 amino acid GFP-tagged Nud1 domain of centriolin was sufficient to induce cytokinesis failure and binucleate formation (Fig. 5e,g) at levels similar to those described for other proteins involved in cytokinesis [27-29]. This occurred in the absence of detectable changes in microtubule organization at any cell cycle stage including telophase when midbodies formed (Fig. 5c,d) and without disrupting endogenous centriolin from centrosomes (Fig. 5f). The results from gene silencing and

protein overexpression support a microtubule-independent mechanism for cytokinesis failure.

The centriolin Nud1 domain interacts with the yeast Bub2p in vitro.

Budding yeast Nud1p anchors the MEN to the spindle pole body through direct interactions with Bub2p and perhaps other MEN components [32,33]. To determine if the centriolin Nud1 homology domain (Fig. 5h) had similar properties we tested its ability to bind Bub2p by directed two-hybrid analysis and immunoprecipitation. Because no vertebrate Bub2p homologue has been unequivocally identified [34] we examined the ability of the centriolin Nud1 domain to interact with yeast Bub2p. Both two-hybrid analysis and immunoprecipitation from yeast cells co-expressing the two proteins revealed a strong and specific interaction between the centriolin Nud1 domain and Bub2p (Fig. 5i,j). No signal was observed when either protein was used alone and no binding was detected between the centriolin Nud1 domain and the budding yeast MEN component Bfa1p, consistent with interactions observations in budding yeast between Nud1p and Bub2p but not Bfa1p [10].

Cleavage failure is observed in Xenopus embryos injected with centriolin antibodies.

A third and final approach was used to examine centriolin function. When affinity-purified anti-centriolin antibodies (Fig. 1b) were microinjected into one cell of two-cell Xenopus embryos [15], the injected cell failed to cleave or cleaved a few times then arrested; uninjected cells or preimmune IgG-injected cells divided normally (Fig. 6a,d). Centriolin antibody-injected cells arrested with two nuclei and two well-organized microtubule asters indicating that karyokinesis and microtubule organization were normal, but cells failed to complete the final event of mitosis, cell cleavage (Fig. 6c). Preimmune IgG-injected cells had a single nucleus with one or two microtubule asters depending on their cell cycle stage, as would be expected for cells that had undergone normal cell cleavage (Fig. 6b). Taken together, the results from gene silencing, antibody injection and protein overexpression in several experimental systems all demonstrate that centriolin plays an important role in the late stages of cytokinesis.

siRNA-induced gene silencing of centriolin causes G1/G0 arrest.

Cytokinesis defects and delays induced by siRNAs targeting centriolin were observed at early times after treatment of RPE-1 cells (24 hours). At later times (48-72 hours post treatment) a reduction in the mitotic index was observed suggesting that the cells were arrested at some other stage of the cell cycle. This was directly tested by treating cells with nocodazole to induce mitotic arrest. Under these conditions, most lamin siRNA-treated control RPE-1 cells arrested in mitosis (71%), while only a small fraction of centriolin siRNA-treated cells arrested at this cell cycle stage (~1%). To determine the cell cycle stage of arrest, cells were analyzed by flow cytometry. In the presence of nocodazole, control cells showed a significant shift from the G1 peak to the G2/M peak (Fig. 7a, red). In contrast, cells treated with siRNAs targeting centriolin did not significantly shift into the G2/M peak in the presence of nocodazole but remained largely in G1 (Fig. 7a, blue). The inability to undergo a nocodazole-induced shift into the G2/M peak was a feature shared by cells driven into G0 by serum starvation (Fig. 7b, blue). Cells were not arrested in S-phase as the proportion of cells in S-phase was slightly decreased in cells silenced for centriolin both in the presence of nocodazole (centriolin, 13%; lamin, 23%) or in its absence (centriolin, 13%; lamin, 19%). These results demonstrate that cells with reduced centriolin arrest prior to S-phase, possibly in G1/S, G1, or G0.

Ki-67 staining was also used to examine the stage of cell cycle arrest. Ki-67 is an antibody directed against a nuclear protein that stains cycling cells or cells arrested in cycle (ex. G1/S, S-phase [35]) but not cells that exit the cell cycle and become quiescent (G0) or undergo differentiation. As expected, nearly all untreated RPE-1 cells or control cells treated with siRNAs targeting GFP or lamins A/C were positive for Ki-67 (Fig. 7c,d). However, most cells with reduced centriolin had undetectable levels of Ki-67 staining (Fig. 7c,d). Taken together, results from mitotic index assays, flow cytometry and Ki-76 staining in RPE-1 cells and HME-1 cells (data not shown) demonstrated that reduction of centriolin levels prevented cells from entering S-phase and appeared to drive them out of cycle into a G0-like state. This cell cycle arrest effectively prevents initiation of additional rounds of centrosome duplication in cells compromised by having

diminished levels of centriolin.

Discussion

In this paper we characterize centriolin, a core centrosome/centriole component whose disruption induces cytokinesis failure and subsequent G1/G0 arrest. The ability of centriolin to perturb these functions may provide a molecular explanation for the cytokinesis defects and G1 arrest previously observed in vertebrate cells following experimental removal of entire centrosomes [6,7]. Moreover, association of centriolin with the maternal centriole may be a molecular requirement for activation of the final stages of cytokinesis, a function recently suggested for the maternal centriole in vertebrate cells [8].

Our data demonstrate that centriolin is required for a late step in cytokinesis, perhaps the final stage of cell cleavage known as abscission. Recent evidence suggests that the maternal centriole is required for three different functions during cytokinesis. Movement of the maternal centriole to the intercellular bridge correlates with bridge narrowing and depolymerization of microtubules within the bridge, and movement away from the bridge correlates with cell abscission [8]. While mitosis and the early events of cytokinesis appear normal in cells with reduced centriolin, including the formation of intercellular bridges with reduced diameter and few/no microtubules, the final abscission event is delayed or never completed. This is perhaps best seen in cells that re-enter mitosis while still interconnected. Under these conditions, the progeny also appear to fail cytokinesis at the final stage of cell abscission and form multicellular syncytia.

The cytokinesis phenotype observed in cells treated with siRNAs targeting centriolin is unique among proteins that function in cytokinesis. The predominant feature in both HeLa cells and RPE-1 cells treated with centriolin-specific siRNAs is the persistent retention of intercellular bridges that keep would-be daughter cells interconnected for extended periods of time. This is in contrast to the phenotype reported for other proteins that cause cytokinesis failure, namely coalescence of dividing cells into binucleate cells. These include bona fide cytokinesis proteins located to the central spindle, regulatory proteins that control this process, microtubule associated proteins and the centrosome protein γ tubulin [27-29,36-39]. Thus, the mechanism of centriolin-induced cytokinesis failure

appears to be distinctly different from that of other vertebrate proteins involved in cytokinesis. Although we observe a low but consistent level of binucleate cells when centriolin levels are reduced, this likely reflects a transient event that is part of a dynamic process. Time-lapse imaging demonstrates that "binucleate" cells ultimately move apart, but can re-coalesce into binucleate cells as they try to complete cytokinesis. Thus, we do not believe that cells treated with centriolin siRNAs accumulate as binucleate cells as seen when bona fide cytokinesis proteins are perturbed. Rather, our data indicate that cells with reduced centriolin remain interconnected by intercellular bridges but ultimately divide to form viable progeny or undergo cell death in the process. Division of cells into viable progeny may not proceed by normal mechanisms but may occur artificially by traction-mediated cytokinesis where cells in culture crawl apart and break in two [40].

Another unique feature of cells with reduced centriolin is their apparent ability to activate apoptosis following long delays in cytokinesis. These cells bleb, round up and lose contact with the substrate, while control cells immediately flatten and crawl apart. This suggests that cells silenced for centriolin may respond to long delays in cleavage by undergoing apoptosis. Alternatively, cells may trigger apoptosis because they cleave artificially by traction-mediated cytokinesis prior to completion of a series of biochemical/regulatory events that may be required for completion of cytokinesis (see [41]). In either case, the apoptotic events are cell cycle specific as they have been observed only at late stages of cytokinesis.

We have not yet determined the precise mechanism of cytokinesis failure following abrogation of centriolin function. Spindle organization, function and duration were not different from control cells. Moreover, microtubule organization appeared normal under three different experimental conditions (gene silencing, Nud1 domain overexpression, centriolin antibodies). Midzone microtubules, midbodies and centrosome nucleation and integrity also appeared normal. A possible clue to the molecular mechanism of cytokinesis failure came from the analysis of the 120 amino acid Nud1 domain of centriolin. We determined that the cytokinesis activity of centriolin resides in this domain which shares homology with yeast proteins involved in microtubule anchoring and

regulation of mitotic exit/cytokinesis, Nud1p and Cdc11p. Like Nud1p, the centriolin Nud1 domain produces defects in the final stage of mitosis and interacts with yeast Bub2p. Importantly, the cytokinesis phenotype is observed without perturbing localization of centriolin to centrosomes, suggesting that the phenotype could be produced by sequestration of other proteins involved in the completion of cytokinesis, perhaps members of a vertebrate pathway similar to the MEN/SIN. Based on these results, we propose a model in which the centriolin Nud1 domain induces cytokinesis failure by disrupting a regulatory pathway in vertebrate cells similar to the yeast MEN and SIN pathways. Unequivocal demonstration of this point will require identification of other members of the pathway. Alternative explanations for the centriolin-induced cytokinesis failure, that do not exclude a role in regulation, are defects in activation of the final cell cleavage event or defects in the incorporation of membrane into the cleavage site [42,43].

Cells that fail in cytokinesis become aneuploid and contain excess centrosomes. Such cells have the potential to contribute to tumorigenesis by inducing further genetic instability through the organization of multipolar spindles by supernumerary centrosomes and by facilitating the accumulation of activated oncogenes and the loss of tumor suppressor genes [44,45]. In this regard, regulation of centriolin activity is critical for ensuring accurate segregation of chromosomes and preventing genetic instability that can potentially drive tumor development.

Our results demonstrate that cells with reduced centriolin ultimately arrest in G1/G0. Previous studies have shown that G1 arrest occurs when entire centrosomes are physically eliminated from vertebrate cells by laser ablation or microsurgery [6,7]. Our results suggest that even minor changes in centrosome composition can effectively inhibit cell cycle progression. However, the mechanism by which cells arrest is unknown. Because our cells experience difficulty in cytokinesis, it is tempting to suggest that G1/G0 arrest is a consequence of improper cytokinesis. However, it has also been suggested that G1 arrest in cells lacking centrosomes may result from the loss of core centrosome components, improper spindle alignment or the presence of excess DNA in

binucleate cells [6,7,14,29]. We are currently testing a model in which loss of core centrosome components activates a checkpoint involved in monitoring "centrosome integrity" and thus prevents centrosome duplication by arresting cells in G1/G0 as we and others have proposed [41,46].

Experimental procedures

Cell culture and transfections

The cells used primarily in this study were diploid, telomerase-immortalized human retinal pigment epithelial cells (hTERT-RPE-1s, Clontech, Palo Alto, CA) [26]. Other cells included HeLa, COS-7, hTERT-HME1 (human mammary epithelia), U2OS and Xenopus tissue culture cells (XTC). All were grown as described (ATCC, Manassas, VA). COS-7 cells were transfected with cDNAs encoding centriolin, the Nud1 domain, GFP and HA as described (Lipofectamine, InVitrogen, Carlsbad, CA).

Antibodies.

Amino acids 268-903 or centriolin were fused with glutathione-S-transferase (GST, Clontech), overexpressed in E. coli and purified as described [15]. Antibodies raised in rabbits were affinity-purified by passing sera over a GST column to remove anti-GST antibodies then over a GST-centriolin column. Antibodies to the following proteins were also used in this study: lamin A/C (Cell Signaling Technology, Beverly, MA), α and γ tubulins, (Sigma, St. Louis, MO), LexA (Santa Cruz, Santa Cruz, CA), GAL4 TAD (Clontech), Ki-67, hemagglutinin (BD Biosciences, Franklin Lakes, NJ).

Immunofluorescence and electron microscopy.

Cells were prepared for immunofluorescence and imaged as described [47] then deconvolved using Metamorph software (Universal Imaging Corp., Downingtown, PA). Fluorescence quantification of centrosomes was performed as described [47]. Unless otherwise noted, all immunofluorescence images are two-dimensional projections of three-dimensional reconstructions to ensure that all stained material was visible in two-dimensional images. Immunogold electron microscopy was performed as described [15] using centrosome fractions from HeLa cells [23] and antibodies to centriolin followed by antibodies coupled to 5 nm gold particles (Amersham, Piscataway, NJ). Pillar cells were prepared and stained as described [24].

Centriolin cloning.

A cDNA of ~ 1.7 kB was identified by screening a human placenta expression library with serum from individuals with scleroderma [15]. The nucleotide sequence was compared with others (blastn) in the human genome database (NCBI) and revealed a sequence with 99% identity on chromosome 9 q34.11-34.13. Genscan predicted a ~7 kb gene comprising 40 exons. PCR primers were used to obtain a ~7 Kb cDNA in a human testes cDNA library. The 5' end, obtained by rapid amplification of cDNA ends (RACE), was identical to the predicted sequence. A full-length HA-tagged centriolin was obtained by inserting an HA tag (YPYDVPDYASL) 5' to the RACE fragment and ligating the HA-centriolin cDNA to the original fragment. The full-length centriolin cDNA contained 6975 nucleotides with an open reading frame of 2325 amino acids and predicted a molecular weight of 269 kD, consistent with the molecular weight of endogenous centriolin (Fig. 1).

Regions flanking the ORF had a translational start (Kozak sequence), polyadenylation sequence, poly-A tail and multiple upstream and downstream stop codons. The construct was inserted into pcDNA 3.1 Zeo (+) (InVitrogen) using BamHI and NotI restriction sites. Centriolin was translated in vitro (TNT, Promega, Madison, WI) and expressed in cultured cells using conventional procedures (Lipofectamine, InVitrogen). Centriolin amino acids 435-623 and 1385-1658 were 24% identical (I)/47% similar (S) and 20% I/41% S to the C-terminal half of TACCs, respectively. Amino acids 879-913 were 40% I/51% S to amino acids 72-106 of human stathmin. Amino acids 126-234 were 31-35% I/47-50% S to Nud1p and Cdc11p.

siRNAs and morpholino antisense.

siRNAs targeting centriolin, lamin A/C and GFP mRNAs were made as complimentary single stranded 19-mer siRNAs with 3' dTdT overhangs (Dharmacon Research, Lafayette, CO), deprotected, annealed and delivered into cells (Oligofectamine, Invitrogen). A 400 μM stock was used to increase the efficiency of gene silencing observed using published stocks. Nucleotides targeted: in centriolin, 117-136 and 145-163, in lamin A/C, 608-630 and in pEGFP-C1 (Clontech), 233-252.

Fluorescein-conjugated morpholino antisense DNA oligonucleotides (Gene Tools, Philomath, OR) targeting the start codon of centriolin (5'-TTTGTTGAGAACCTTTCTTCATTGC) were introduced into cells using the EPEI agent (Gene Tools). The inverse sequence was used as control.

Time lapse imaging.

HeLa cells plated on coverslips (25 mm diameter) were treated with siRNAs targeting centriolin for 50 hours. They were placed in a chamber (PDMI-2, Harvard Apparatus, MA) in complete medium with CO_2 exchange (0.5 liters min^{-1}) at 37° C. Cells were imaged every 10 minutes for 12-20 hours using a 20X or 40 X phase contrast lens with a green interference filter on an inverted microscope (Olympus IX-70, Melville, NY). Images were captured on a CoolSnap HQ CCD camera (Roper Scientific, Tucson, AZ) and concatenated using Metamorph software (Universal Imaging, Downington, PA).

Primary cilium formation and microtubule nucleation.

Primary cilia were induced by culturing hTERT-RPE-1 cells in medium with 0.25% serum for 48 hrs and identified using the GT335 antibody raised to polygluatamylated α, β tubulins [4]. Microtubule nucleation was performed as described [48] by treated with nocodazole (1 mg ml^{-1}) for 1 hour at 37°C, fixing cells at various times after washing out drug then staining for microtubules.

RT-PCR.

Centriolin mRNA levels were assayed by reverse transcription polymerase chain reaction using 10 µl mRNA (OneStep RT-PCR, Qiagen, Valencia, CA); α tubulin served as an internal control in the same reaction. Alls products were sequenced. Primers: specific for human α tubulin, forward 5'-AAAGATGTCAATGCTGCC-3' and reverse 5'-TCCTCTCCTTCTTCCTCAC-3'; for centriolin, forward 5'-CCATCATCATCTCACTCTC-3' and reverse 5'-CTTCCCTAACCATACTGG-3'.

Yeast two hybrid analysis and immunoprecipitations.

A fragment containing amino acids 127-233 of centriolin was cloned into EcoR I and Sal I sites of pGADT7 (Clontech) to produce a fusion with the GAL4 transactivation domain (TAD). Constructs pEG202 (LexA), pGP69 (LexA-*BUB2*), pGP122 (LexA-*BFA1*) and the yeast strain SGY37 were from Elmar Schiebel. SGY37, which contains a *LacZ* reporter gene under control of a LexA operator, was transformed with plasmid DNA using LiAc [49] and transformants selected for on dropout medium. We used semi-quantitative β–galactosidase assays [50] and more quantitative β-galactosidase assays with CPRG (chlorophenol red-β-D-galactopyranoside, Roche, Indianapolis, IN) as a substrate (Clontech Yeast Protocols Handbook). Co-immunopreciptation of LexA and GAL4 TAD fusion proteins were carried out as described [50].

Flow cytometry. Cells treated with siRNAs for 50-70 hours were treated with 100 ng ml^{-1} for 12 hours, removed from plates and fixed in methanol. Cells stained with propidium iodide were analyzed by flow cytometry (FACSCAN, Becton Dickinson, Burlingame CA) using Flojo software (Tree Star, Inc., Stanford University).

Accession numbers. Centriolin, AF513978, lamin A/C, X03444.

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References

1. Doxsey, S. J. Re-evaluating centrosome function. *Nature Reviews in Molecular Biology* **2**, 688-699 (2001).
2. Chretien, D., Buendia, B., Fuller, S. D. & Karsenti, E. Reconstruction of the centrosome cycle from cryoelectron micrographs. *J Struct Biol* **120**, 117-33. (1997).
3. Piel, M., Meyer, P., Khodjakov, A., Rieder, C. L. & Bornens, M. The respective contributions of the mother and daughter centrioles to centrosome activity and behavior in vertebrate cells. *J Cell Biol* **149**, 317-30. (2000).
4. Bobinnec, Y. et al. Centriole disassembly in vivo and its effect on centrosome structure and function in vertebrate cells. *J. Cell Biol.* **143**, 1575-1589 (1998).
5. Rappaport, R. Establishment of the mechanism of cytokinesis in animal cells. *Int Rev Cytol* **105**, 245-81 (1986).
6. Hinchcliffe, E. H., Miller, F. J., Cham, M., Khodjakov, A. & Sluder, G. Requirement of a centrosomal activity for cell cycle progression through G1 into S phase. *Science* **291**, 1547-50. (2001).
7. Khodjakov, A. & Rieder, C. L. Centrosomes enhance the fidelity of cytokinesis in vertebrates and are required for cell cycle progression. *J Cell Biol* **153**, 237-42. (2001).
8. Piel, M., Nordberg, J., Euteneuer, U. & Bornens, M. Centrosome-dependent exit of cytokinesis in animal cells. *Science* **291**, 1550-3. (2001).
9. Bardin, A. J. & Amon, A. Men and sin: what's the difference? *Nat Rev Mol Cell Biol* **2**, 815-26. (2001).
10. Pereira, G. & Schiebel, E. The role of the yeast spindle pole body and the mammalian centrosome in regulating late mitotic events. *Curr Opin Cell Biol* **13**, 762-9. (2001).
11. McCollum, D. & Gould, K. L. Timing is everything: regulation of mitotic exit and cytokinesis by the MEN and SIN. *Trends Cell Biol* **11**, 89-95. (2001).
12. Glotzer, M. Animal cell cytokinesis. *Annu Rev Cell Dev Biol* **17**, 351-86 (2001).
13. Guertin, D. A., Trautmann, S. & McCollum, D. Cytokinesis in eukaryotes. *Microbiol Mol Biol Rev* **66**, 155-78. (2002).
14. Andreassen, P. R., Lohez, O. D., Lacroix, F. B. & Margolis, R. L. Tetraploid State Induces p53-dependent Arrest of Nontransformed Mammalian Cells in G1. *Mol Biol Cell* **12**, 1315-28. (2001).
15. Doxsey, S. J., Stein, P., Evans, L., Calarco, P. & Kirschner, M. Pericentrin, a highly conserved protein of centrosomes involoved in microtubule orgainzation. *Cell* **76**, 639-650 (1994).
16. Lupas, A., VanDyke, M., and Stock, J. Predicting coiled-coils from protein sequences. *Science* **252**, 1162-1164 (1991).
17. Lee, M. J., Gergely, F., Jeffers, K., Peak-Chew, S. Y. & Raff, J. W. Msps/XMAP215 interacts with the centrosomal protein D-TACC to regulate microtubule behaviour. *Nat Cell Biol* **3**, 643-9. (2001).
18. Andersen, S. S. Spindle assembly and the art of regulating microtubule dynamics by MAPs and Stathmin/Op18. *Trends Cell Biol* **10**, 261-7. (2000).

19. Guasch, G. et al. FGFR1 is fused to the centrosome-associated protein CEP110 in the 8p12 stem cell myeloproliferative disorder with t(8;9)(p12;q33). *Blood* **95**, 1788-96. (2000).
20. Lange, B. M. & Gull, K. A molecular marker for centriole maturation in the mammalian cell cycle. *J. Cell Biol.* **130**, 919-927 (1995).
21. Mogensen, M. M., Malik, A., Piel, M., Bouckson-Castaing, V. & Bornens, M. Microtubule minus-end anchorage at centrosomal and non-centrosomal sites: the role of ninein. *J Cell Sci* **113**, 3013-23. (2000).
22. Vorobjev, I. A. & Chentsov, Y. S. Centrioles in the cell cycle. I. Epithelial cells. *J Cell Biol* **93**, 938-49. (1982).
23. Blomberg, M. & Doxsey, S. J. Rapid isolation of centrosomes. *Meth. Enzymol.* **298**, 228-238 (1998).
24. Mogensen, M. M., Mackie, J. B., Doxsey, S. J., Stearns, T. & Tucker, J. B. Centrosomal deployment of gamma-tubulin and pericentrin: Evidence for a microtubule-nucleating domain and a munis-end docking domain in certain mouse epithelial cells. *Cell Motil. Cytoskel.* **36**, 276-290 (1997).
25. Elbashir, S. M. et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494-8. (2001).
26. Morales, C. P. et al. Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nat Genet* **21**, 115-8. (1999).
27. Mollinari, C. et al. PRC1 is a microtubule binding and bundling protein essential to maintain the mitotic spindle midzone. *J Cell Biol* **157**, 1175-86. (2002).
28. Matuliene, J. & Kuriyama, R. Kinesin-like protein CHO1 is required for the formation of midbody matrix and the completion of cytokinesis in mammalian cells. *Mol Biol Cell* **13**, 1832-45. (2002).
29. Meraldi, P., Honda, R. & Nigg, E. A. Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53-/- cells. *Embo J* **21**, 483-92. (2002).
30. Murphy, S. M., Urbani, L. & Stearns, T. The mammalian gamma-tubulin complex contains homologues of the yeast spindle pole body components spc97p and spc98p. *J Cell Biol* **141**, 663-74. (1998).
31. Fry, A. M. et al. C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. *J Cell Biol* **141**, 1563-74. (1998).
32. Gruneberg, U., Campbell, K., Simpson, C., Grindlay, J. & Schiebel, E. Nud1p links astral microtubule organization and the control of exit from mitosis. *Embo J* **19**, 6475-88. (2000).
33. Pereira, G., Hofken, T., Grindlay, J., Manson, C. & Schiebel, E. The Bub2p spindle checkpoint links nuclear migration with mitotic exit. *Mol Cell* **6**, 1-10. (2000).
34. Cuif, M. H. et al. Characterization of GAPCenA, a GTPase activating protein for Rab6, part of which associates with the centrosome. *Embo J* **18**, 1772-82. (1999).
35. Gerdes, J. et al. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* **133**, 1710-5. (1984).

36. Seong, Y. S. et al. A spindle checkpoint arrest and a cytokinesis failure by the dominant- negative polo-box domain of Plk1 in U-2 OS cells. *J Biol Chem* **277**, 32282-93. (2002).
37. Kaiser, B. K., Zimmerman, Z. A., Charbonneau, H. & Jackson, P. K. Disruption of Centrosome Structure, Chromosome Segregation, and Cytokinesis by Misexpression of Human Cdc14A Phosphatase. *Mol Biol Cell* **13**, 2289-300. (2002).
38. Manabe, R., Whitmore, L., Weiss, J. M. & Horwitz, A. R. Identification of a Novel Microtubule-Associated Protein that Regulates Microtubule Organization and Cytokinesis by Using a GFP-Screening Strategy. *Curr Biol* **12**, 1946-51. (2002).
39. Shu, H. B., Li, Z., Palacios, M. J., Li, Q. & Joshi, H. C. A transient association of gamma-tubulin at the midbody is required for the completion of cytokinesis during the mammalian cell division. *J Cell Sci* **108**, 2955-62. (1995).
40. Burton, K. & Taylor, D. L. Traction forces of cytokinesis measured with optically modified elastic substrata. *Nature* **385**, 450-4. (1997).
41. Doxsey, S. J. Centrosomes as command centres for cellular control. *Nat Cell Biol* **3**, E105-8. (2001).
42. Finger, F. P. & White, J. G. Fusion and fission: membrane trafficking in animal cytokinesis. *Cell* **108**, 727-30. (2002).
43. Thompson, H. M., Skop, A. R., Euteneuer, U., Meyer, B. J. & McNiven, M. A. The Large GTPase Dynamin Associates with the Spindle Midzone and Is Required for Cytokinesis. *Curr Biol* **12**, 2111-7. (2002).
44. Pihan, G. A. et al. Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression. *Cancer Res* **61**, 2212-9. (2001).
45. Doxsey, S. Duplicating dangerously: linking centrosome duplication and aneuploidy. *Mol Cell* **10**, 439-40. (2002).
46. Quintyne, N. J. & Schroer, T. A. Distinct cell cycle-dependent roles for dynactin and dynein at centrosomes. *J Cell Biol* **159**, 245-54. (2002).
47. Dictenberg, J. et al. Pericentrin and gamma tubulin form a protein complex and are organized into a novel lattice at the centrosome. *J. Cell Biol.* **141**, 163-174 (1998).
48. Purohit, A., Tynan, S. H., Vallee, R. & Doxsey, S. J. Direct interaction of pericentrin with cytoplasmic dynein light intermediate chain contributes to mitotic spindle organization. *J. Cell Biol.* **147**, 481-491 (1999).
49. Ito, H., Fukuda, Y., Murata, K. & Kimura, A. Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**, 163-8. (1983).
50. Schramm, C., Janke, C. & Schiebel, E. Molecular dissection of yeast spindle pole bodies by two hybrid, in vitro binding, and co-purification. *Methods Cell Biol* **67**, 71-94 (2001).

Figure legends

Figure 1 **Centriolin is a ~270 kD coiled coil protein localized to mature centrosomes and the midbody. (a)** Schematic showing centriolin coiled coil regions (boxes), noncoiled regions (lines) and domains homologous to budding and fission yeast Nud1p and Cdc11p, human stathmin and human and Drosophila TACC proteins. **(b) First panel:** ^{35}S(Methionine)-labeled HA-tagged centriolin produced by in vitro translation of cDNA and resolved directly (Cen) or after immunoprecipitation with HA antibodies (Cen IP); empty vector (Vec). **Second panel:** Western blots probed with anti-HA antibody showing overexpressed HA-tagged centriolin (HA-Cen) and its absence from cells overexpressing β galactosidase (β gal). **Third and forth panels:** Centrosome fractions prepared from HeLa cells and Xenopus cells (XTC) blotted with antibodies to centriolin (Cen) or preimmune sera (PreI). Arrowheads show position of centriolin. In XTC cells, the bands below centriolin appear to be degradation products as they are sometimes observed in protein fractions produced by in vitro translation or overexpression. Bars represent positions of molecular weight markers (x10^3). **(c)** Immunofluorescence images of endogenous centriolin in RPE-1 cells at different cell cycle stages. **Upper panels:** Merged images of centriolin (green), γ tubulin (red) and nuclei (blue) from cells in **G1, G2, proM (prometaphase), M (metaphase) and anaphase (A). Insets:** higher magnifications of centrosomes stained for γ tubulin (left panels) and centriolin (right panels). Centriolin localization to one of the two centrosomes is demonstrated most clearly in the G2 cell. **Middle panels (Telo early) and bottom panels (Telo late)** show separate images of γ tubulin staining (left) and centriolin (right). γ tubulin marks both mother and daughter centrioles, which are separated in some cases. Centriolin staining is confined to one of two centrioles, which sometimes appears at the intercellular bridge (Telo early). Centrioles lacking centriolin are indicated by arrowheads in right panels. At later stages of telophase (Telo late), centriolin is also on the midbody. All immunofluorescence images (here and elsewhere) are two-dimensional projections of three-dimensional reconstructions to ensure that all stained material is visible. C, centriole, MB, midbody. Bar in bottom right of c, 10 μm for all except insets, 3 μm.

Figure 2 **Centriolin is localized to maternal centrioles and noncentrosomal sites of microtubule anchoring. (a)** HA-tagged centriolin overexpressed in COS-7 cells localizes to the centrosome (anti-HA, green) at the convergence of microtubules (red, anti-α tubulin). **(b)** An RPE-1 cell immunostained with an antibody to polygluamylated tubulin (GT335) [4] to label centrioles and the primary cilium (red), and for centriolin (green) which is localized to the maternal centriole (m) associated with the primary cilium (yellow in merge) but not on the daughter centriole (d). n, nucleus. **(c-e)** Electron micrographs showing specific immunogold labeling of centriolin on subdistal appendages found on maternal (**e**, bottom, M) but not daughter centrioles (**e**, top, D). **c** and **d** are longitudinal and cross-sections, respectively, through maternal centrioles and e is a longitudinal section through both centrioles. Arrowheads show striations characteristic of subdistal appendages. **(f, g)** Centriolin is found at noncentrosomal sites of microtubule anchoring in pillar cells of the mouse cochlea [24] (arrows). **f**, Schematic representation of pillar cell. **g**, Centriolin immunofluorescence staining overlaid with phase contrast image. Centrosome and associated cilium is shown schematically at top of cell in f. Bars: a, g, 10 μm; b, 2 μm; c-e, 100 nm.

Figure 3 **RPE-1 cells treated with siRNAs targeting centriolin retain persistent intercellular connections and fail in cytokinesis. (a)** RT-PCR analysis shows that centriolin mRNA is reduced in RPE-1 cells treated with centriolin-specific siRNAs (top right) but is unaffected in cells treated with siRNAs targeting lamins A/C (top left, sequence identity confirmed). Control (α tubulin) RT-PCR was performed in the same reaction mixtures with centriolin and lamin (bottom panels). **(b)** Immunofluorescence images (top right, γ tubulin, red and centriolin, green) and quantification of centriolin levels in centrosomes from cells treated with siRNAs as indicated. The **top right panel** shows a cell with undetectable centriolin at the centrosome/centriole and the **top left panel** shows a cell that is unaffected by the treatment and stains for centriolin (green/white). Graph shows the average centriolin fluorescence intensity/pixel at individual centrosomes (bars) in cells treated with lamin or centriolin siRNAs. The centrosome fluorescence in most centriolin siRNA-treated cells (83%) was below the

lowest values observed in control cells. **(c)** Graph showing a dramatic increase in the percentage of cells in telophase/cytokinesis following siRNA targeting of centriolin (~70-fold). n, total number of cells counted (c,d). **(d)** Graph showing the increased percentage of binucleate cells in HeLa, U2OS and RPE-1 cell lines following treatment with centriolin siRNAs (4 to 15-fold greater than controls). In c and d, values represent data from single experiments representative of 3-4 experiments. Time analyzed: HeLa, 48 hours (h), 72 h, U2OS, 72 h, RPE-1, 24 h. **(e-l)** Microtubule organization **(e-j)** and microtubule nucleation **(k,l)** are not detectably altered in cells treated with centriolin siRNA **(f,h,j,l)** compared with cells treated with lamin siRNA **(e,g,i,k).** Arrows indicate position of centrosomes; no centrosome staining is observed in centriolin siRNA-treated cells. Insets in k and l are enlargements of centriolin staining at centrosomes (or microtubule convergence sites) at arrows. Cells in interphase (e and f, lower cell), prometaphase/metaphase (proM/M, g,h) and telophase (i,j) were labeled for microtubules (red), centrosomes (green/yellow) and nuclei (blue). MB, midbody. **(m,n)** Cells treated with siRNAs targeting centriolin were stained for microtubules (red) and DNA (blue). Arrowheads indicate contiguous connections between two or more cells. In **m**, three interconnected cells form a syncytium. In **n**, one daughter of an interconnected pair of cells has re-entered mitosis (M). Bar: in n, 5 μm for e-j, 3.5 μm for k and l and insets, and 15 μm for m and n.

Figure 4 **Time-lapse images of HeLa cells treated with centriolin siRNAs reveals unique cytokinesis defects. (a)** A cell treated with control siRNAs targeting lamin moves apart, forms visible midbodies (arrow) and completes the final cleavage event with normal timing (1-3 hours after metaphase). **(b-d)** Centriolin siRNA-treated cells remain attached for extended periods of time through persistent intercellular bridges and sometimes do not show visible midbodies. **b,** A cell that remains attached by a long intercellular bridge for at least 8 hours. The cell cleaves, both daughter cells round up and at least one appears to undergo apoptotic cell death (upper cell, 7:20, extensive blebbing, decrease in size). **c,** A dividing cell that has not completed cleavage re-enters the next mitosis. One cell rounds up and is drawn to the other. The other rounds up and both undergo the early stages of cytokinesis to form a total of four cells; these 'progeny' often

remain attached by intercellular bridges forming syncytia. **d,** Cell showing three failed attempts at cell cleavage over a 9.5 hour time period. **(e)** Cells treated with siRNAs targeting centriolin progress from nuclear envelope breakdown (NEB) to anaphase with normal timing, similar to lamin siRNA controls. Vertical bars represent recordings from single cells (e, f). **(f)** Centriolin siRNA treated cells are delayed in cytokinesis (~70%) compared with control lamin siRNA-treated cells, a value consistent with a 70-80% silencing efficiency. Results represent recordings of individual cells from several independent experiments. Times in hours and minutes are included in upper portion of each panel in a-d. Bar in d, 10 μm for all.

Figure 5 **Cells expressing centriolin fail in cytokinesis, as do cells expressing the centriolin Nud1 domain that interacts with yeast Bub2p. (a)** COS-7 cell overexpressing HA-tagged centriolin (left inset) showing persistent microtubule bundles in the intercellular bridge during cytokinesis (main panel, α tubulin staining) despite reformation of the nucleus and decondensation of chromatin (DNA, right inset). **(b)** Control COS-7 cell (expressing HA alone, left inset) at a similar cell cycle stage based on nuclear morphology (inset, right) show a narrow intercellular bridge and diminished microtubule polymer (main panel), characteristic of cells that have reformed nuclei and decondensed DNA. **(c-f)** COS-7 cells expressing a GFP-tagged Nud1 domain of centriolin (insets, c,d, green; DNA, blue) have normal intercellular bridges (**d,** telophase, arrowhead) and normal microtubule organization (**c,** interphase) as in controls. However, the cells often become binucleate (**e**, yellow, blue nuclei) by a mechanism that does not involve disruption of the endogenous centrosome-associated centriolin (**f**, inset right shows enlargement of centrosome, inset left shows transfected cell). **(g)** Quantitative analysis showing that HA-centriolin and the GFP-tagged Nud1 domain both induce significant binucleate cell formation compared to controls (HA, GFP). Values are from a single experiment and representative of 3 experiments. Bar in d, 7 μm for all. n, total number of cells counted. **(h)** Alignment of the centriolin Nud1 domain with the yeast Nud1p and Cdc11p proteins. **(i)** Direct yeast two hybrid analysis demonstrates an interaction between the human Nud1 domain with one of the two components implicated in GTPase activating protein (GAP) activity, Bub2p, but not Bfa1p. The blue colony in

blue box (middle bottom) and increased β galactosidase activity (Bar 2 of graph) demonstrate a specific interaction between the human centriolin Nud1 domain (hNud1) and yeast Bub2p. Bar 1, LexA- BUB2 x transactivation domain (TAD), Bar 2, LexA-BUB x TAD-hNud1, Bar 3, LexA-BFA1 x TAD, Bar 4, TAD-hNud1 x LexA-BFA1, Bar 5, TAD-hNud1 x LexA. **(j)** Specific co-immunoprecipitation of HA-tagged hNud1 and Lex A-tagged yeast Bub2p from yeast cells. Co-precipitation was observed using antibodies to either protein and only when both were co-expressed (top middle panel in each group). Lane 1, TAD-HA-hNud1 x LexA, lane 2, TAD-HA-hNud1xBub2p-LexA, lane 3, TAD-HAxBub2p-LexA.

Figure 6. **Cytokinesis failure in Xenopus embryos injected with centriolin antibodies.** **(a)** Xenopus two cell embryos injected into one cell with ~50 nl anti-centriolin antibodies (2 mg ml^{-1} affinity purified) fail to cleave (arrows) while noninjected cells (side opposite arrows) and cells injected with control rabbit IgG (2 mg ml^{-1}, top) cleave normally. **(b,c)** Immunofluorescence images showing two microtubule asters near a single nucleus (N) in a cell from an embryo injected with control IgG, **(b)**, and two nuclei and two asters in a cell from a centriolin antibody-injected cell (**c,** microtubules stained with anti-alpha tubulin). **(d)** Quantification of results from injection experiments showing ~8-fold increase in cleavage failure (representative of 3 experiments). n, number embryos examined. Bar in b, 10 μm for b, c.

Figure 7 **siRNAs targeting centriolin induce G1/G0 arrest.** **(a)** RPE-1 cells treated with centriolin siRNAs (blue) do not shift into the G2/M peak following nocodazole treatment as seen for control lamin siRNA-treated cells (red). **(b)** The inability of cells treated with centriolin siRNAs to shift into the G2/M peak is similar to that observed in serum-starved cells (blue, serum starved; red, not starved). **(c)** Ki-67 staining of control cells (left cell, green) occurs together with centriolin staining (left cell, red at arrow); both are gone in centriolin siRNA-treated cells (right, arrowhead). **(d)** Cells treated with siRNAs targeting centriolin lack Ki-67 staining (~70%), while most control cells stain positively (GFP siRNAs). a,b,d are representative experiments from 5 experiments for a, 2 for b, 3 for d. Bar in d, 7.5 μm. n, total number cells counted.

Supplementary material: Time-lapse images of cells treated with siRNAs targeting lamin (control) or centriolin. (see Fig. 4 and Fig. 4 legend for additional details)

Movie #1 (lamin.control.siRNA) Cell treated with control siRNAs targeting lamin forms intercellular bridge, forms visible midbodies (phase dense structure between dividing cells) and completes the final cleavage event with normal timing (1-3 hours after metaphase).

Movie #2 (Centriolin.siRNA) A cell that remains attached by a long intercellular bridge for at least 8 hours. The cell cleaves, both daughter cells round up and at least one appears to undergo apoptotic cell death--upper cell shows extensive blebbing, rounding up, decrease in size and ultimately loses substrate attachment. Note: apparent membrane ruffling in intercellular bridge.

Movie #3 (Centriolin.siRNA) A dividing cell that remains attached by a long intercellular bridge re-enters the next mitosis. One cell rounds up and is drawn to the other. The other rounds up and both undergo the early stages of cytokinesis to form a total of four cells; these 'progeny' often remain attached by intercellular bridges forming syncytia (some connections can be seen in upper cell).

Figure 1

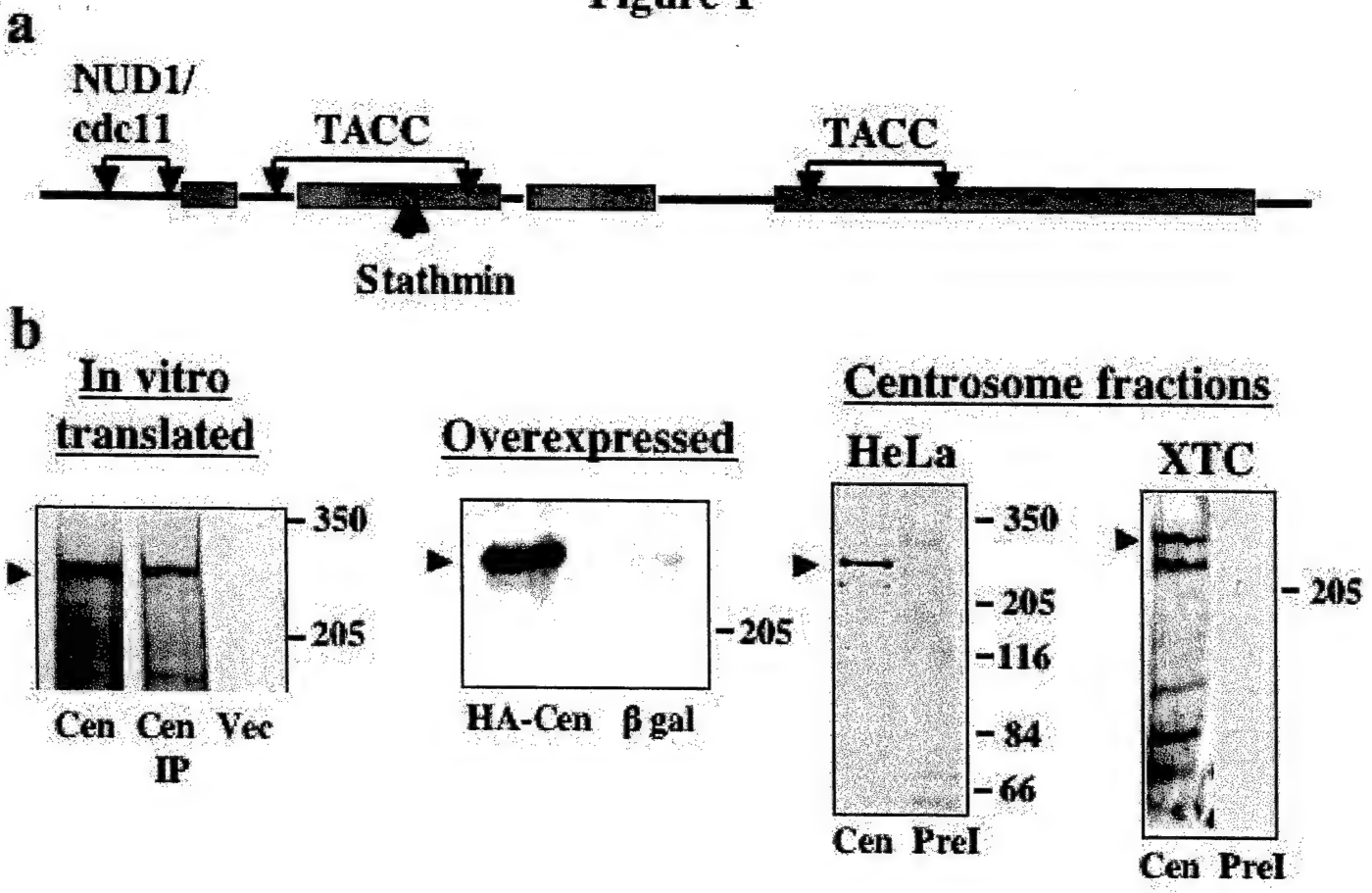

a
NUD1/
cdc11
TACC
TACC
Stathmin
b
In vitro
translated
Overexpressed
Centrosome fractions
HeLa
XTC
350
205
205
350
205
116
84
66
205
Cen Cen Vec
IP
HA-Cen β gal
Cen PreI
Cen PreI
c
G1
G2
proM
M
A
γ tubulin
centriolin
1C
1C
Telo early
2C
γ tubulin
centriolin
2C
MB
1C
1C
MB
Telo late

Figure 2

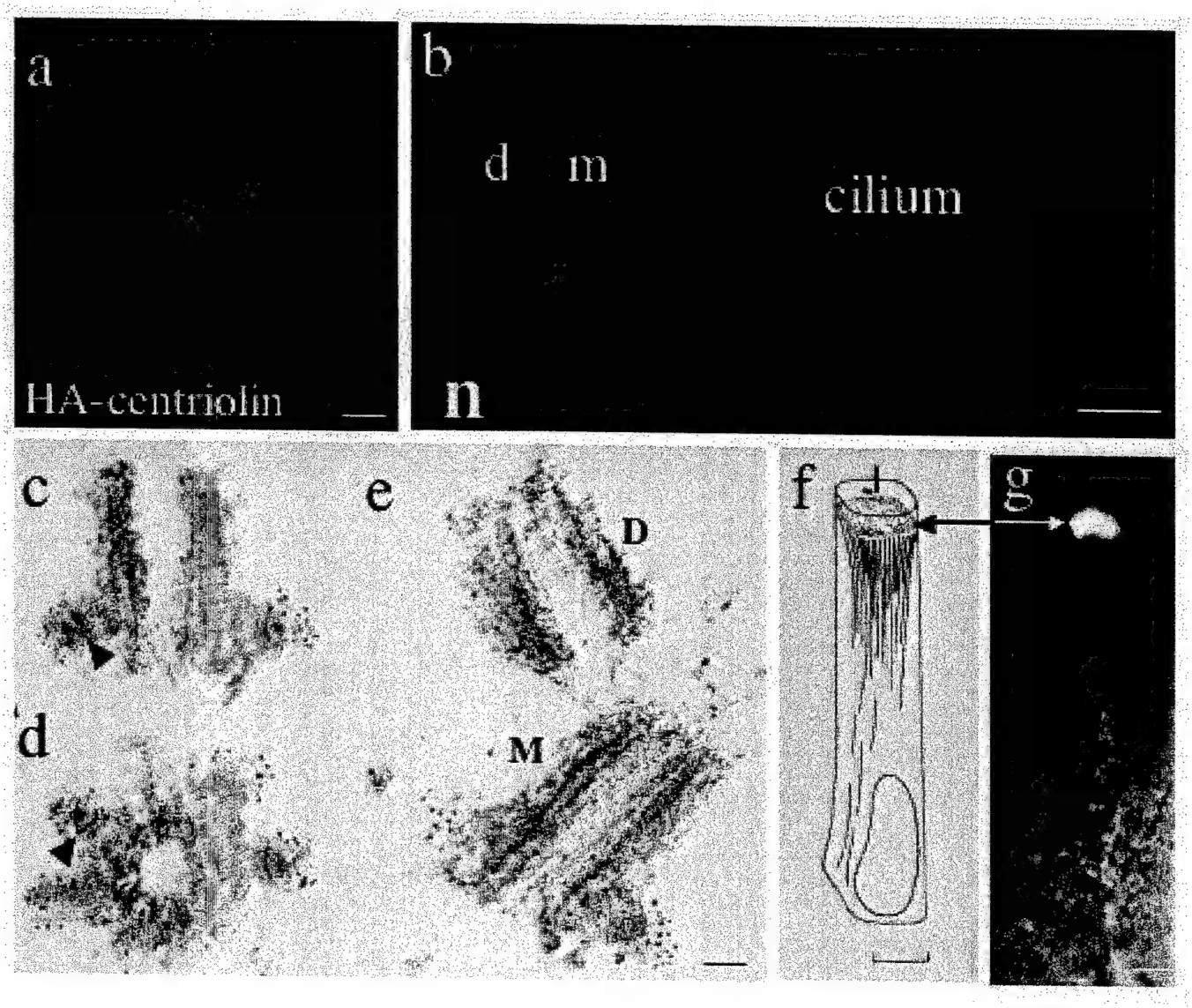


Figure 3

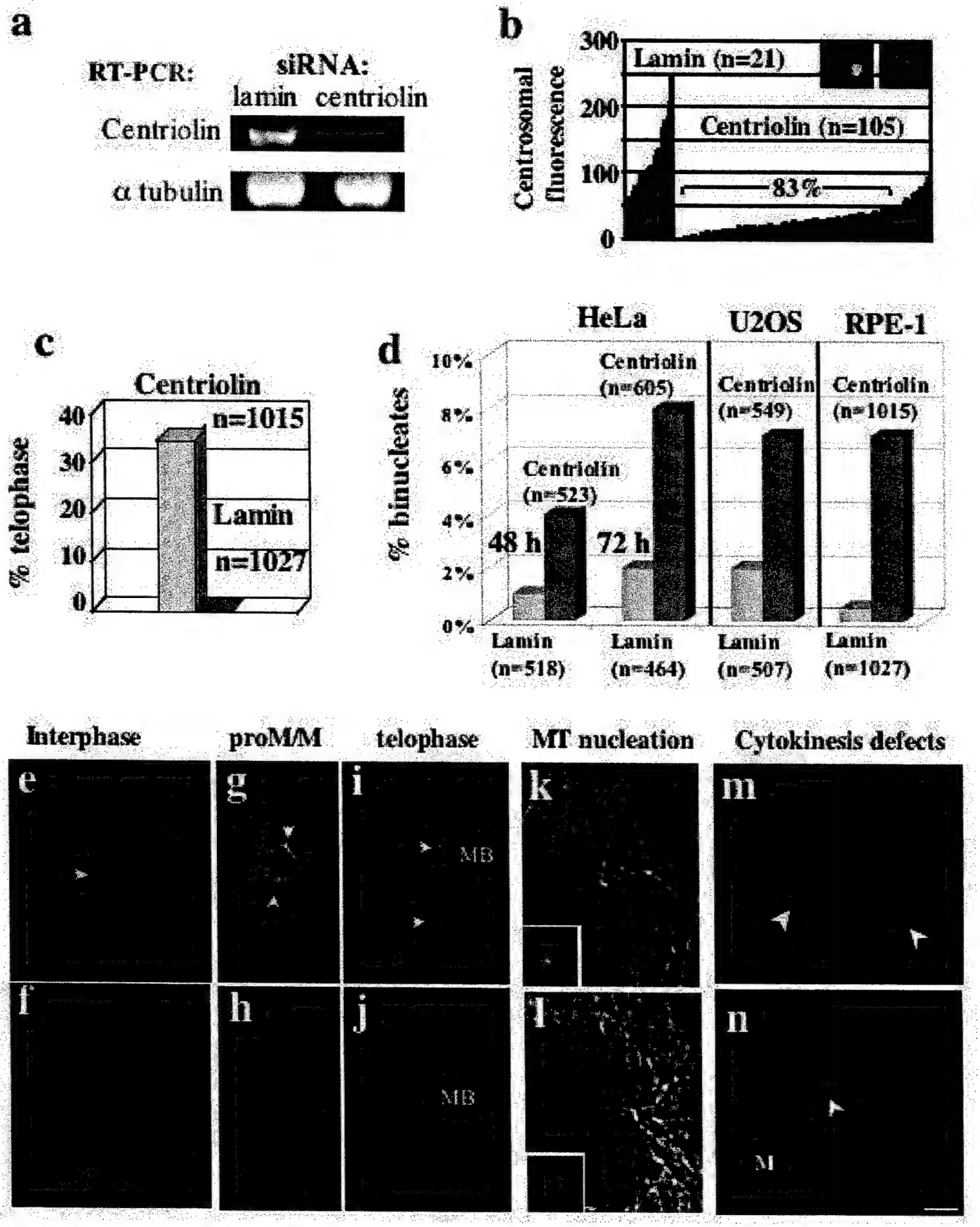

a
RT-PCR:
siRNA:
lamin centriolin
Centriolin
α tubulin
b
Centrosomal fluorescence
300
200
100
0
Lamin (n=21)
Centriolin (n=105)
83%
c
Centriolin
% telophase
40
30
20
10
0
n=1015
Lamin
n=1027
d
HeLa
U2OS
RPE-1
% binucleates
10%
8%
6%
4%
2%
0%
Centriolin (n=605)
Centriolin (n=523)
48 h
72 h
Centriolin (n=549)
Centriolin (n=1015)
Lamin (n=518)
Lamin (n=464)
Lamin (n=507)
Lamin (n=1027)
Interphase
proM/M
telophase
MT nucleation
Cytokinesis defects
e
f
g
h
i
MB
j
MB
k
l
m
n
M

Figure 4

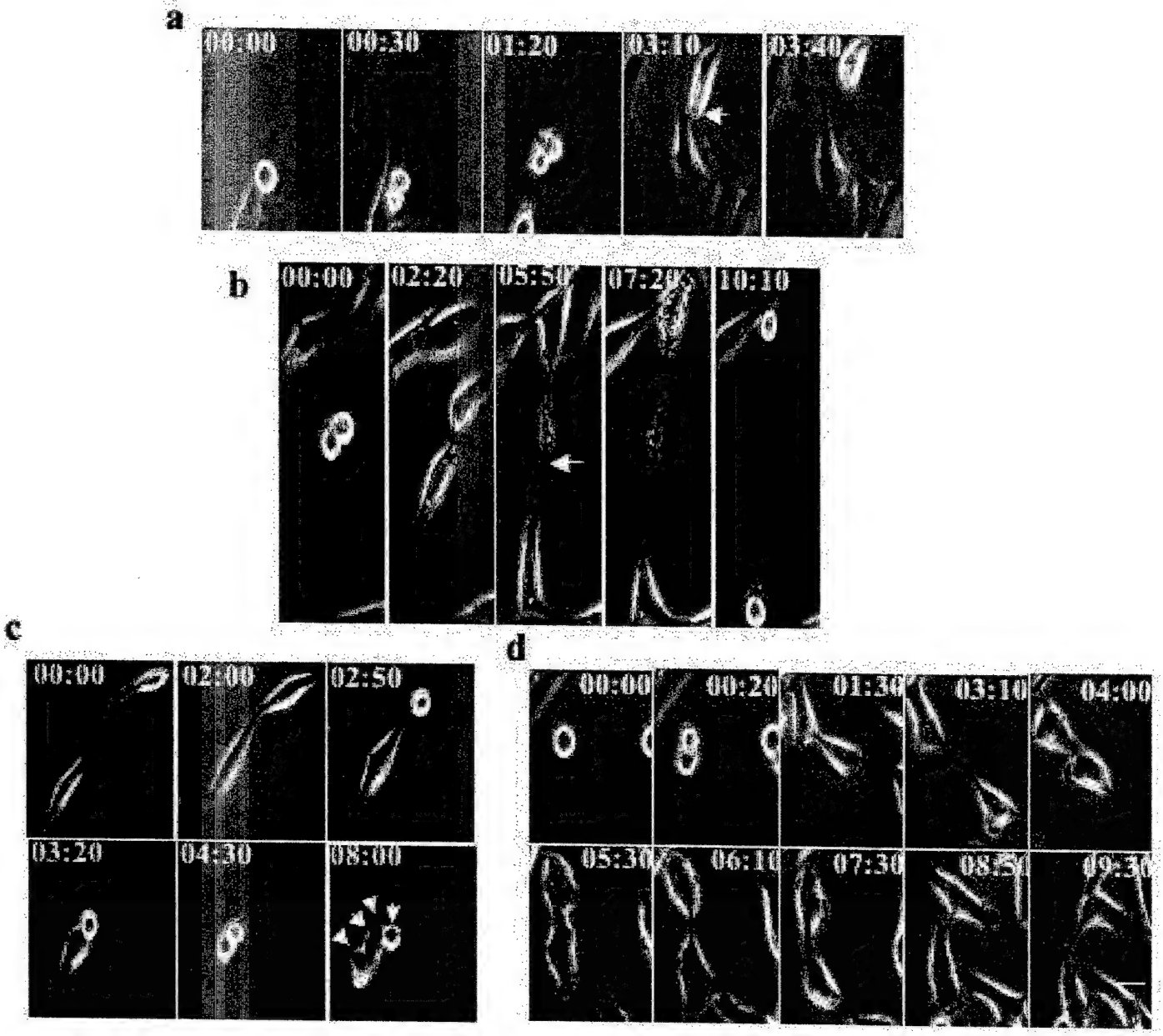
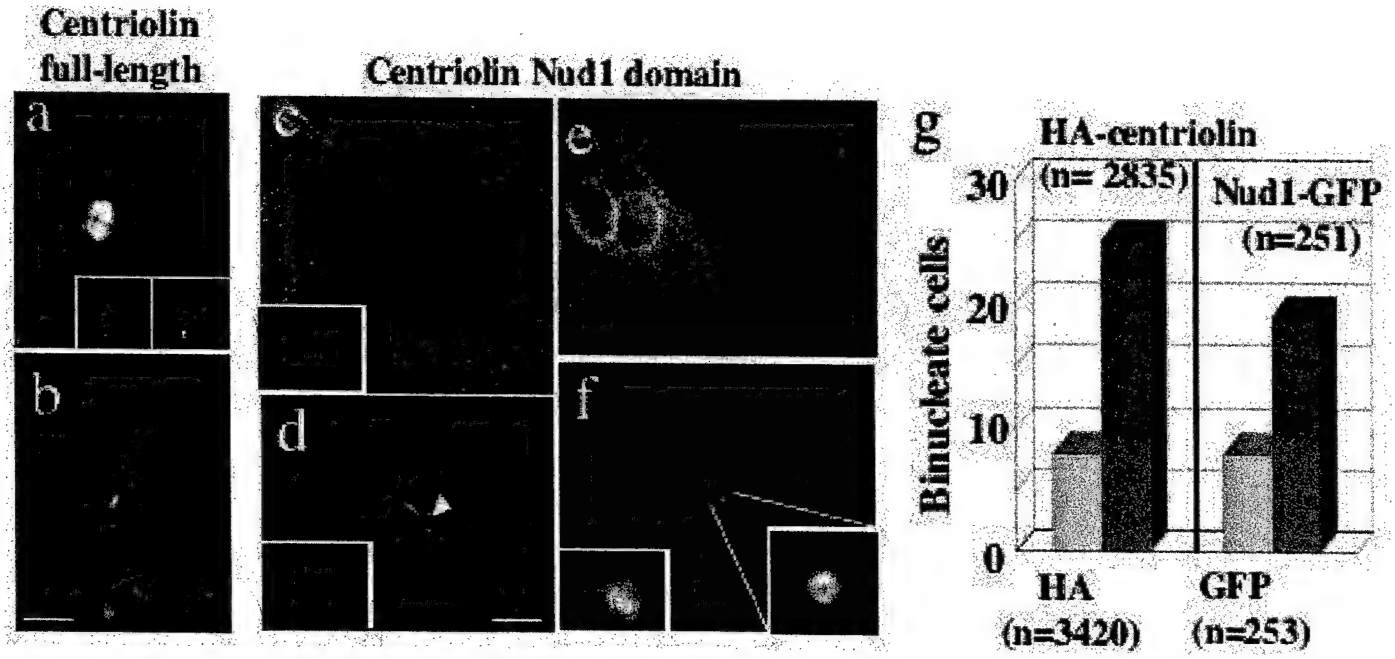
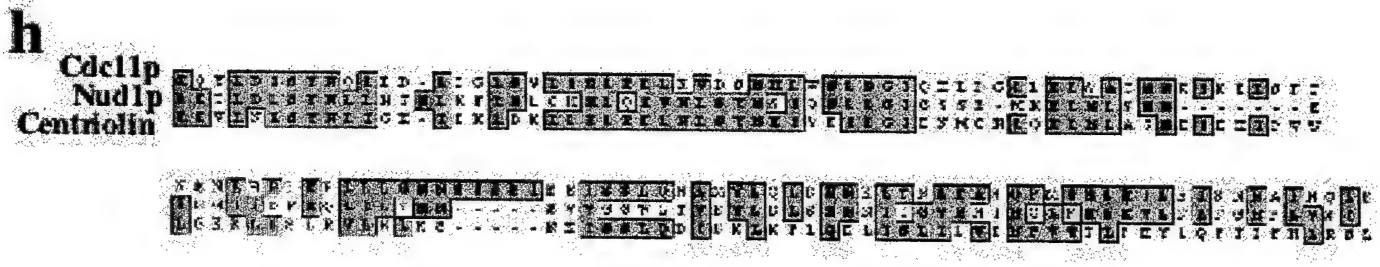
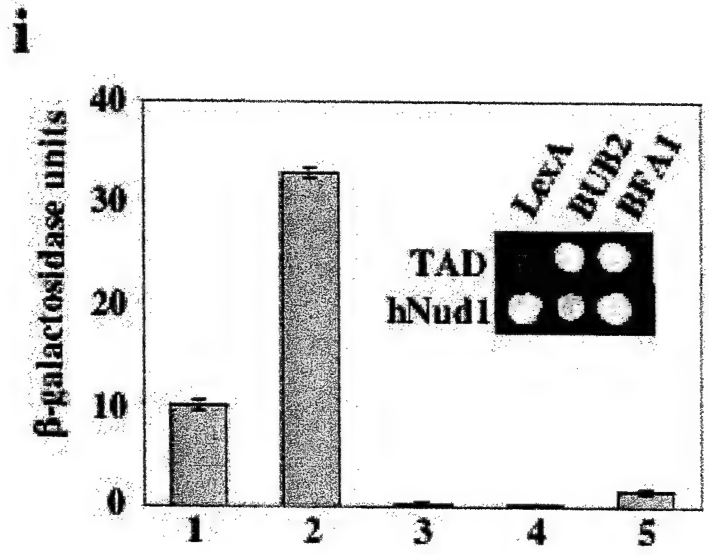

a
00:00
00:30
01:20
03:10
03:40
b
00:00
02:20
05:50
07:20
10:10
c
00:00
02:00
02:50
03:20
04:30
08:00
d
00:00
00:20
01:30
03:10
04:00
05:30
06:10
07:30
08:50
09:30
e
NEB to anaphase
Time [min]
100
90
80
70
60
50
40
30
20
10
0
Lamin
Centriolin
f
Metaphase to cleave
Time [min]
1400
600
500
400
300
200
100
0
Centriolin
Lamin
71%

Figure 5


Centriolin
full-length
Centriolin Nud1 domain
a
b
c
d
e
f
g
HA-centriolin
(n= 2835)
Nud1-GFP
(n=251)
Binucleate cells
30
20
10
0
HA
(n=3420)
GFP
(n=253)


h
Cdc11p
Nud1p
Centriolin


i
β-galactosidase units
40
30
20
10
0
1
2
3
4
5
LexA
BUB2
BFA1
TAD
hNud1

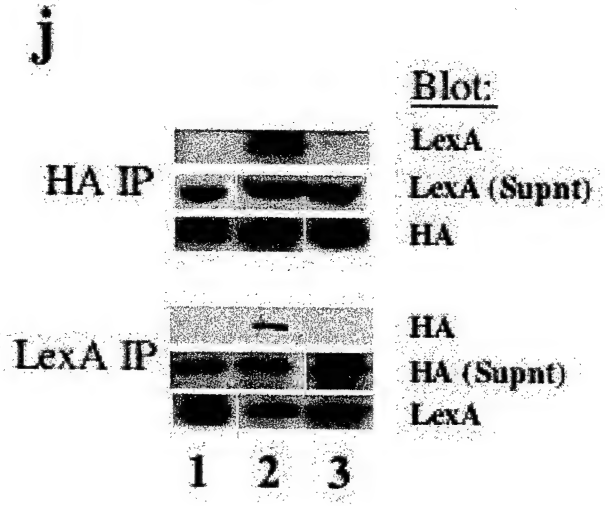

j
Blot:
HA IP
LexA
LexA (Supnt)
HA
LexA IP
HA
HA (Supnt)
LexA
1
2
3

Figure 6

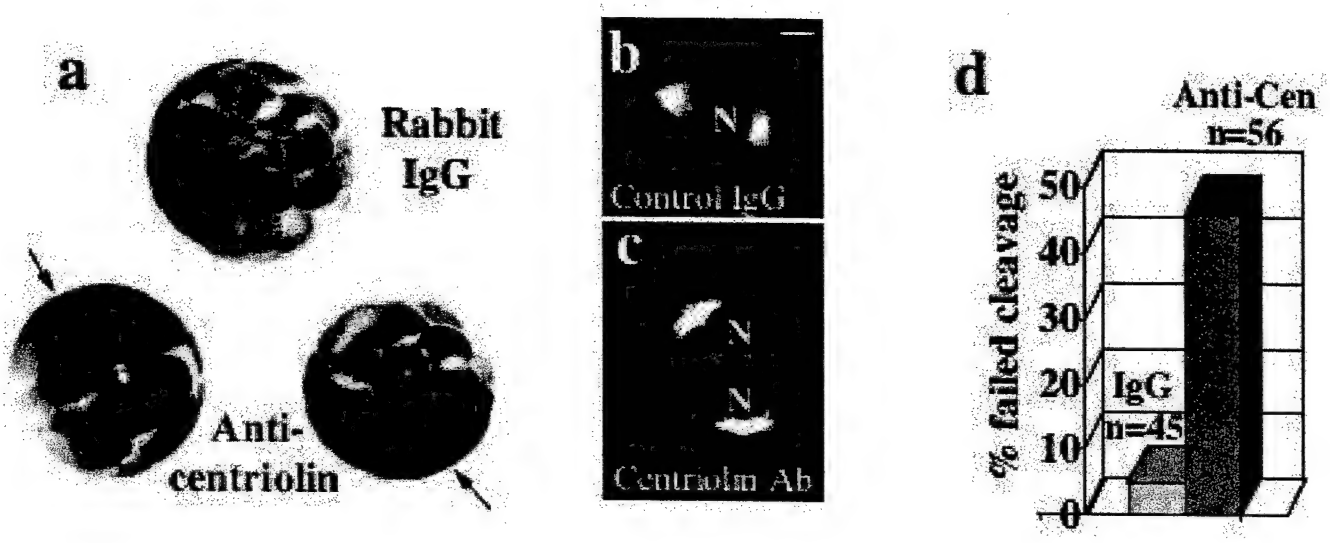
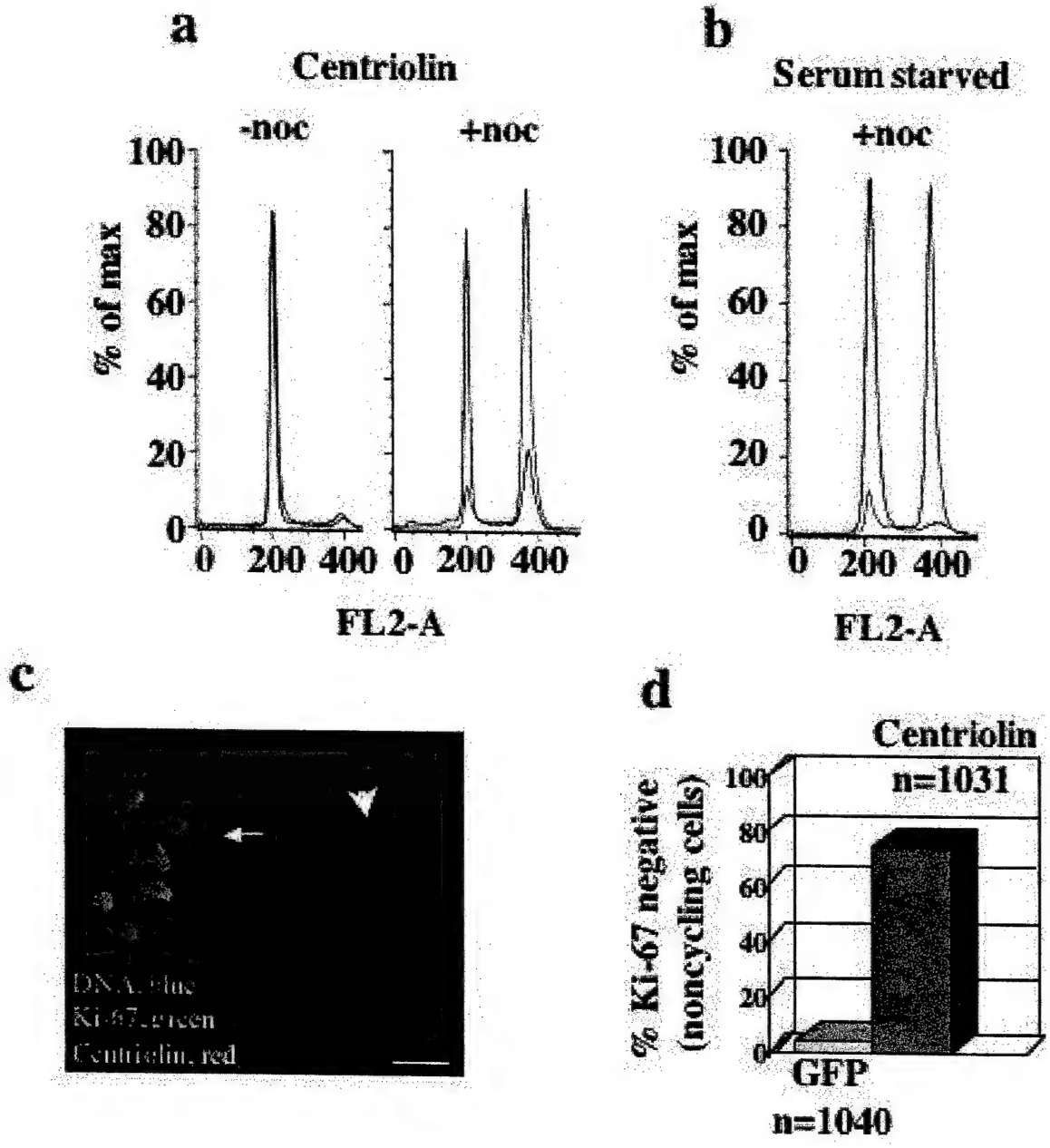

a
Rabbit IgG
Anti-centriolin
b
N
Control IgG
c
N
N
Centriolin Ab
d
Anti-Cen n=56
% failed cleavage
50
40
30
20
10
0
IgG n=45

Figure 7


a
Centriolin
-noc
+noc
100
80
60
40
20
0
% of max
0 200 400 0 200 400
FL2-A
b
Serum starved
+noc
100
80
60
40
20
0
% of max
0 200 400
FL2-A
c
DNA, blue
Ki-67, green
Centriolin, red
d
Centriolin
n=1031
% Ki-67 negative (noncycling cells)
100
80
60
40
20
0
GFP
n=1040

whimsy which confirms the autonomous nature of this RNA sensor. Similar 5′ UTR sensors may be hiding in the shadows of biology, as yet unknown but responding to other environmental or cellular cues. Some may even be molecular fossils of structures dating back to an RNA world. PrfA is only one of a remarkable class of molecules that measure the immeasurable, but it will surely bring intense heat to the rapidly expanding universe of gene expression control.

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Selected Reading

Brown, A.J., Sun, L., Feramisco, J.D., Brown, M.S., and Goldstein, J.L. (2002). Mol. Cell *10*, 237–245.

Froehlich, A.C., Liu, Y., Loros, J.J., and Dunlap, J.C. (2002). Science *297*, 815–819.

He, Q., Cheng, P., Yang, Y., Wang, L., Gardner, K.H., and Liu, Y. (2002). Science *297*, 840–843.

Hoe, N.P., and Goguen, J.D. (1993). J. Bacteriol. *175*, 7901–7909.

Hurme, R., and Rhen, M. (1998). Mol. Microbiol. *30*, 1–6.

Johansson, J., Mandin, P., Renzoni, A., Chiaruttini, C., Springer, M., and Cossart, P. (2002). Cell *110*, 551–561.

Katayama, S., Matsushita, O., Tamai, E., Miyata, S., and Okabe, A. (2001). FEBS Lett. *509*, 235–238.

Liu, P.C.C., and Thiele, D.J. (1999). J. Biol. Chem. *274*, 17219–17225.

Madrid, C., Nieto, J.M., Paytubi, S., Falconi, M., Gualerzi, C.O., and Juarez, A. (2002). J. Bacteriol. *184*, 5058–5066.

Seegmiller, A.C., Dobrosotskaya, I., Goldstein, J.L., Ho, Y.K., Brown, M.S., and Rawson, R.B. (2002). Dev. Cell *2*, 229–238.

Raney, A., Law, G.L., Mize, G.J., and Morris, D.R. (2002). J. Biol. Chem. *277*, 5988–5994.

Tell, G., Pines, A., Paron, I., D'Elia, A., Bisca, A., Kelley, M.R., Manzini, G., and Damante, G. (2002). J. Biol. Chem. *277*, 14564–14574.

Duplicating Dangerously: Linking Centrosome Duplication and Aneuploidy

Centrosomes are far more fascinating than the first explorers of this organelle a century ago could ever have imagined. Recent evidence indicates that deregulation of centrosome duplication affects centrosome number and promotes aneuploidy, features characteristic of human tumors.

Centrosomes are the major microtubule organizing structures in vertebrate cells. They nucleate and organize microtubules for numerous cellular functions and act as centers for integrating regulatory activities including cell cycle progression, checkpoint control, and stress responses. In dividing vertebrate cells, centrosomes form the poles of mitotic spindles where they direct segregation of chromosomes and play an important role in the final stages of cell division or cytokinesis (see Hinchcliffe and Sluder, 2001).

Defects in centrosome function or number can adversely affect spindle function and cytokinesis and induce genetic instability. While bipolar spindles with two centrosomes segregate chromosomes equally into two daughter cells, increased centrosome numbers can produce multipolar spindles that promote chromosome missegregation. Failure to duplicate or separate centrosomes in mitosis can induce formation of monopolar spindles that do not properly direct cell division and induce polyploidy. Polyploid cells with multiple centrosomes can also arise from cytokinesis failure resulting from centrosome dysfunction. The vital role of centrosomes in partitioning the genome led to the hypothesis by Boveri one hundred years ago that these organelles have the potential to contribute to genetic instability and human tumor development. Recent papers suggest that misregulation of centrosome duplication can alter centrosome number and possibly cause genetic instability (Chen et al., 2002; Fisk and Winey, 2001; Okuda et al., 2000).

A centrosome in the G1 phase of the cell cycle has two microtubule barrels, the centrioles, surrounded by a protein matrix that nucleates microtubules. As cells progress through the cell cycle, each centriole acts as a template to initiate growth of a new daughter centriole. The duplication process is completed by mitosis when the duplicated centrosomes separate and organize the mitotic spindle. Centrosome duplication is regulated by protein complexes containing the centrosome-associated cell cycle kinase CDK2 and cyclin E or A; ubiquitin-mediated proteolysis and other kinases also play a role (see Hinchcliffe and Sluder, 2001). It is certain that a better understanding of centrosome duplication will emerge as phosphorylation targets of CDKs are identified.

One intriguing downstream target of CDKs was identified in the September issue of *Developmental Cell* by Dynlacht and coworkers (Chen et al., 2002). This novel 110 kDa protein, CP110, was localized to centrosomes, and its expression coincided with timing of CDK2 activation and centrosome duplication. CP110 function was tested by reducing protein levels with small interfering RNAs (siRNAs). Cells were then examined following treatment with hydroxyurea, a drug that inhibits S phase progression but supports multiple rounds of duplication. While multiple rounds of centrosome duplication were completed in the presence of nonsense siRNAs, duplication was inhibited in cells treated with CP110-specific siRNAs. To test the role of CDK-dependent phosphorylation of CP110 in centrosome duplication, cell lines were constructed that stably expressed a mutant form of CP110 that could not be phosphorylated. Surprisingly, centrosome numbers and presumably centrosome duplication did not appear aberrant in cells expressing the mutant CP110. However, expression of the mutant did induce tetraploidy.

It is clear that CP110 is involved in centrosome duplication, but how the protein affects duplication and ploidy is not obvious. For example, while CP110 silencing blocks centrosome duplication in S phase-arrested cells, it has no apparent effect on duplication in wild-type cells that progress normally through the cell cycle. One explanation for this result is that residual protein remaining in siRNA-treated cells (~10% of total) supports a single round of duplication in cycling cells but is insufficient to support multiple rounds of duplication in S phase-arrested cells. Additional experiments in systems that allow complete removal of the protein should provide insights into the role of CP110 in centrosome duplication in cycling cells, as will overexpression of the protein, which might induce centrosome amplification (see below). Another unresolved question is how apparently stable populations of cycling tetraploid cells with normal centrosome numbers develop from expression of the CP110 phosphorylation mutant. One might expect that such cells would lose the ability to duplicate their centrosomes and cease to proliferate. It is argued that these cells may represent clonal derivatives of the original cells that survived the otherwise lethal effects of centrosome dysfunction and polyploidy. In any case, a clearer picture of how the phosphorylation mutant affects centrosome duplication and ploidy will require analysis of cells at earlier stages of CP110 expression using inducible promoters.

Two other downstream phosphorylation targets of CDK2-cyclin E that affect centrosome duplication have been identified. The mouse ortholog of budding yeast Mps1p is a centrosome-associated kinase stabilized by CDK2 activity and thus appears to function jointly with CDK2 (Fisk and Winey, 2001). Overexpression of murine Mps1 induces reduplication of centrosomes during S phase arrest, while a kinase-deficient mutant blocks centrosome duplication altogether. However, the human Mps1 does not localize to centrosomes or affect centrosome duplication and appears to function primarily in control of the spindle checkpoint (Stucke et al., 2002). Additional studies performed under the same conditions will be required to understand the function of Mps1. Another target of CDK2-cyclin E is nucleophosmin (NPM/B23) (Okuda et al., 2000). This protein associates with unduplicated centrosomes and appears to be released from centrosomes following phosphorylation by CDK2-cyclin E. Inhibition of NPM/B23 in mammalian cells by antibodies or overexpression of a mutant protein that lacks the CDK2-cyclin E phosphorylation site, inhibits centrosome duplication in normal cycling cells. Future studies will determine whether CP110, Mps1, and NPM/B23 act together or independently to control centrosome duplication.

Centrosome defects have now been discovered in many human tumors although the mechanism by which they arise is unknown (Lingle et al., 2002; Pihan et al., 2001). They could develop through primary defects in the centrosome cycle as discussed above, or by delays in cell cycle progression (e.g., S phase), cell division failure (Meraldi et al., 2002), or ectopic assembly of centrosome proteins into acentriolar spindle poles (Pihan et al., 2001). It is still unclear whether centrosome defects contribute to human cancer or if they are a consequence of the disease. Their presence at early stages of human tumor development (Lingle et al., 2002; Pihan et al., 2001) and the ability of some centrosome proteins to induce tumor-like features upon overexpression are consistent with a role for centrosomes in tumor progression. There is still no clear genetic evidence to indicate that centrosome abnormalities initiate or exacerbate tumor progression. A stronger link between centrosome defects and cancer will require identification of genetic mutations in the CDK targets described above and in other molecules that directly affect centrosomes in human tumors.

Human cancer arises from a stepwise accumulation of genetic changes (Hahn and Weinberg, 2002). It is possible that centrosome defects arise in or influence only cells that are mutated in pathways associated with tumorigenesis such as cell cycle progression, apoptosis, and cell proliferation. Centrosome defects could act as epigenetic modifiers of the genome and together with genetic mutations provide a powerful driving force for increased genetic instability (Pihan and Doxsey, 1999). This condition could accelerate accumulation of alleles carrying prooncogenic mutations and loss of alleles containing wild-type tumor suppressor genes, features characteristic of the most prevalent human cancers. The consequence of these events would be to create a larger pool of genetically altered cells from which to spawn clonal populations with greater survival potential and other cancer-related qualities. Given the complexities of the cancer cell and of centrosome duplication and function, it may be some time before we fully understand the contribution of the centrosome to human tumorigenesis.

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Selected Reading

Chen, Z., Indjeian, V.B., McManus, M., Wang, L., and Dynlacht, B.D. (2002). Developmental Cell *3*, 339–350.

Fisk, H.A., and Winey, M. (2001). Cell *106*, 95–104.

Hahn, W.C., and Weinberg, R.A. (2002). Nat. Rev. Cancer *2*, 331–341.

Hinchcliffe, E.H., and Sluder, G. (2001). Genes Dev. *15*, 1167–1181.

Lingle, W.L., Barrett, S.L., Negron, V.C., D'Assoro, A.B., Boeneman, K., Liu, W., Whitehead, C.M., Reynolds, C., and Salisbury, J.L. (2002). Proc. Natl. Acad. Sci. USA *99*, 1978–1983.

Meraldi, P., Honda, R., and Nigg, E.A. (2002). EMBO J. *21*, 483–492.

Okuda, M., Horn, H.F., Tarapore, P., Tokuyama, Y., Smulian, A.G., Chan, P.K., Knudsen, E.S., Hofmann, I.A., Snyder, J.D., Bove, K.E., and Fukasawa, K. (2000). Cell *103*, 127–140.

Pihan, G.A., and Doxsey, S.J. (1999). Sem Cancer Res. *9*, 289–302.

Pihan, G.A., Purohit, A., Wallace, J., Malhotra, R., Liotta, L., and Doxsey, S.J. (2001). Cancer Res. *61*, 2212–2219.

Stucke, V.M., Sillje, H.H., Arnaud, L., and Nigg, E.A. (2002). EMBO J. *21*, 1723–1732.

RE-EVALUATING CENTROSOME FUNCTION

Stephen Doxsey

Over the past 100 years, the centrosome has risen in status from an enigmatic organelle, located at the focus of microtubules, to a key player in cell-cycle progression and cellular control. A growing body of evidence indicates that centrosomes might not be essential for spindle assembly, whereas recent data indicate that they might be important for initiating S phase and completing cytokinesis. Molecules that regulate centrosome duplication have been identified, and the expanding list of intriguing centrosome-anchored activities, the functions of which have yet to be determined, promises continued discovery.

INTERPHASE
The period between two mitotic divisions.

CENTRIOLES
Open-ended cylinders, comprised of nine sets of triplet microtubules linked together and containing appendages on the outside and protein assemblies and sometimes vesicles on the inside. Centrosomes usually contain two centrioles.

MICROTUBULE
A hollow tube, 25 nm in diameter, formed by the lateral association of 13 protofilaments, which are themselves polymers of α- and β-tubulin subunits.

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Centrosomes were named during the late 1800s by Theodor Boveri, on the basis of their central position in the cell[1]. At that time, the centrosome had been observed as a small focus of phase-dense material, surrounded by a larger region of less phase density. Even then, it was apparent that centrosomes focused the ends of thin cytoplasmic fibres in INTERPHASE cells and bipolar spindles in mitotic cells.

Today, we know that the densely staining structures seen by Boveri are CENTRIOLES; the undifferentiated cytoplasm is the surrounding pericentriolar material; and the thin filaments are MICROTUBULES (FIG. 1). However, 100 years after the discovery of centrosomes, our understanding of their structure, composition and function is still in its infancy. This article discusses selected issues, mainly about animal cell centrosomes, although important contributions about the molecular organization and function of centrosomes come from work on the yeast equivalent, the SPINDLE POLE BODY[2–4].

Centrosome organization

Centrosomes are unusual in that they are the only non-membranous organelles in most vertebrate cells. They occupy a tiny volume (1–2 μm^3), near the centre of the cell, and they are usually in close proximity to the nucleus. In most vertebrate cells, centrosomes are composed of two main substructures — centrioles and pericentriolar material (FIG. 1). Centrioles are strikingly symmetrical barrel-shaped structures, ~0.5 μm long and ~0.2 μm in diameter. Their symmetry is derived from the nine sets of triplet microtubules that make up the barrel, together with other elements (FIG. 1). Each centrosome has two centrioles that lie at right angles to one another and in close proximity at one end (proximal). One centriole has additional appendages at the end farthest from the other centriole (distal) and is called the mother or maternal centriole.

The pericentriolar material usually surrounds both centrioles and is the site of microtubule nucleation[5]. The pericentriolar material seems to be an interconnected meshwork of fibres and protein aggregates that forms a matrix or lattice. This matrix seems to be highly dynamic and is composed of a high proportion of COILED-COIL proteins. Elements of the matrix provide a scaffold for anchoring proteins that are involved in microtubule nucleation[6–8] and other activities.

The exact function of centrioles is still unclear. It has been known for some time that cells of higher plants, some meiotic cells and eggs in some embryonic systems lack centrioles. These cells form acentriolar microtubule-organizing centres (MTOCs), similar in appearance and composition to the nucleation-competent pericentriolar material of centrosomes[1]. In cells that contain them, centrioles can elongate the pre-existing microtubules at the distal end to produce a single, non-motile PRIMARY CILIUM[9]. The function of the primary cilium is unknown but defects in these structures in cells of the collecting duct of the kidney

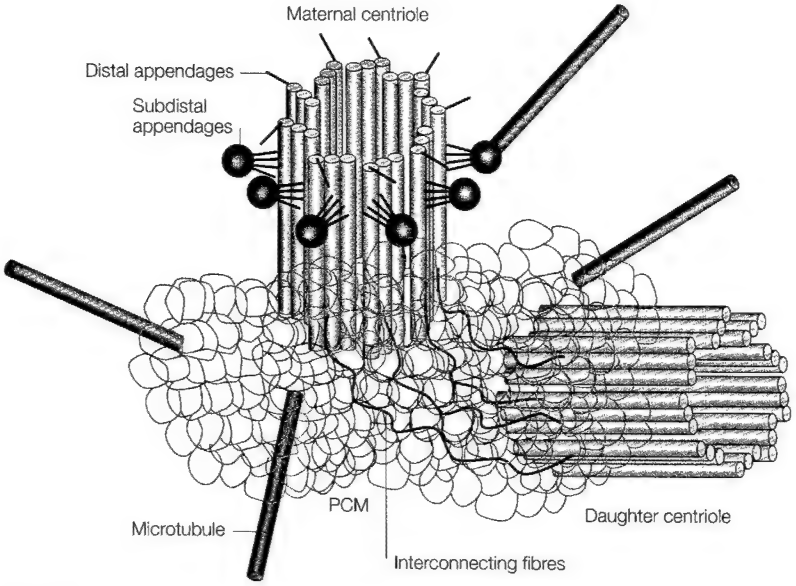

Figure 1 | **Centrosome structure.** A pair of centrioles is shown, each with ninefold symmetry owing to the nine triplet microtubules. Each centriole has pericentriolar material that nucleates microtubules around the ends closest to one another. Only the maternal centriole has two sets of extra appendages, distal and subdistal; the latter seems to anchor microtubules. A series of interconnecting fibres, different from the pericentriolar material (PCM), links the closest ends of the two centrioles.

seem to contribute to polycystic kidney disease[10]. For this reason, the primary cilium has been implicated in essential cellular functions, such as metering of solution flow or sensing ion concentrations in these (and perhaps other) cells[10].

Basal bodies are cousins to centrioles that lack pericentriolar material and form motile cilia and flagella, also by elongation of pre-existing microtubules. Basal bodies of sperm act as templates for the organization of maternal components of the egg after fertilization. The inability of human sperm basal bodies to recruit maternal centrosome components has been implicated in fertilization failures. Moreover, it has been suggested that basal bodies might be lost or damaged during preparation of the nuclei that are used for intracytoplasmic sperm injection (ICSI) and for *in vitro* cloning of vertebrates, and could therefore contribute to the low success rates of these procedures[11,12].

Centrioles, like basal bodies, seem to be involved in the recruitment and organization of centrosome components in somatic cells: their disruption by antibodies against stabilized centriolar microtubules induces dispersion of associated pericentriolar material[13]. An additional role for centrioles in cell-cycle progression has recently been postulated and is discussed in later sections on CYTOKINESIS and the G1 to S transition.

Centrosome-mediated microtubule events

Microtubule nucleation. Centrosomes are the main site of microtubule nucleation in most cells. If cells are treated with drugs that depolymerize microtubules and are then washed, microtubules grow back primarily from centrosomes. Centrosome-nucleated microtubules are polarized with their rapidly growing (plus) ends in the cytoplasm and their slow-growing (or minus) ends anchored at the centrosome. The geometry of these microtubules is regular, each having 13 protofilaments that are organized into a tubular structure[14]. By contrast, microtubules grown *in vitro* with high concentrations of tubulin have a variable number of protofilaments. This suggests the presence of a template for microtubule assembly at the centrosome.

One template for microtubule nucleation at the centrosome is a ring-shaped multiprotein complex containing γ-tubulin — a protein that is very similar to the α- and β-tubulins but is found primarily at centrosomes and does not incorporate into microtubules[7,8,15]. These γ-tubulin ring complexes (γTuRCs) are also found in the cytoplasm, but their function is unknown[16,17]. They might be involved in cytoplasmic microtubule nucleation or capping[18], or they might simply be a source of nucleating complexes that are recruited to centrosomes when increased microtubule nucleation is required[3,19,20]. Budding yeast have a smaller, simpler, γ-tubulin complex[3] whereas other organisms have both large and small γ-tubulin complexes[21,22]. The small γ-tubulin complex and even γ-tubulin alone can nucleate microtubules, albeit with lower efficiencies[22,23]. The three proteins that make up the small yeast complex — Spc97, Spc98 and Tub4 — are conserved among other organisms[24,25], indicating that they probably have essential functions in microtubule nucleation.

Recent advances in the structure and function of the γTuRC indicate that it might be a template for microtubule nucleation and that it caps microtubule minus ends[18,26,27]. γTuRCs are dome-shaped on one side and have repeating subunits on the other, which are thought to be small γ-tubulin complexes. On the basis of these biochemical and structural data, a model has been proposed for the structure of the γTuRC[27], where several small γ-tubulin subcomplexes are organized into a ring by other members of the γTuRC. The γ-tubulin molecules within the small complexes seem to nucleate microtubule protofilaments perpendicular to the ring, and lateral interactions between protofilaments mediate formation of the tubular structure.

An alternative structure has been proposed for the γ-tubulin complex, in which a short segment composed of γ-tubulin subcomplexes provides a preformed protofilament[28]. In this protofilament model, γ-tubulin complexes would be organized in a curved, ring-like structure similar to the γTuRC. However, each γ-tubulin complex would interact laterally with an α/β-dimer to form microtubule protofilaments parallel to — rather than perpendicular to — the string of γ-tubulin subunits. This would straighten the ring of γ-tubulin complexes and produce a sheet of α/β-tubulin protofilaments that would subsequently fold into a tube, producing a microtubule[29]. Additional studies will be required to determine which of the two models is used for microtubule nucleation.

Little is known about how γ-tubulin complexes are recruited to, and anchored at, centrosomes in

SPINDLE POLE BODY
The acentriolar microtubule-organizing centre of yeast and diatoms. It is a plaque-like structure that is embedded in the nuclear membrane that faces the cytoplasm on one side and the nuclear interior on the other.

COILED COIL
A protein domain that forms a bundle of two or three α-helices. Whereas short coiled-coil domains are involved in protein interactions, long coiled-coil domains forming long rods occur in structural or motor proteins.

PRIMARY CILIUM
A single, probably non-motile, cilium that grows from the maternal centriole of the centrosome in most cell types.

CYTOKINESIS
The process of cytoplasmic division.

MITOSIS
The process of nuclear division.

ASTER
A radial array of microtubules focused on and usually nucleated by centrosomes or aggregates of centrosome/spinlde pole proteins.

S VALUE
(Sedimentation coefficient.) A standardized value, describing migration of molecules/particles under a centrifugal force.

mammalian cells. They can nucleate microtubules *in vitro*, but they require additional factors to assemble onto centrosomes[21,25]. In budding yeast spindle pole bodies, Spc72 in the inner (nuclear) plaque and Spc110 in the outer (cytoplasmic) plaque anchor γ-tubulin complexes[30,31]. The Asp (abnormal spindle) protein in *Drosophila melanogaster*, together with isolated γTuRCs, can restore microtubule nucleation to salt-stripped centrosomes, indicating that this protein might provide the centrosome anchor for γ-tubulin during MITOSIS[32]. Mammalian and *Xenopus laevis* Spc110-related proteins have been identified by a monoclonal antibody approach[33], but it is not known whether they can mediate assembly of γ-tubulin complexes or bind to the human homologues of Spc97/Spc98 (GCP2/GCP3).

Pericentrin has been implicated in the recruitment of γTuRCs to centrosomes in vertebrate cells[6]. Recent studies show that pericentrin binds to GCP2 and/or GCP3 of the γTuRC by yeast two-hybrid and co-immunoprecipitation experiments (W. Zimmerman, J. Sillibourne and S.D., unpublished observations). The GCP2/GCP3-binding domain of pericentrin and a pericentrin antibody both precipitate endogenous γ-tubulin complex components from *Xenopus* and cell extracts, and both inhibit ASTER formation and the recruitment of γ-tubulin to sperm centrioles in *Xenopus* extracts. A larger isoform of pericentrin — called pericentrin B or kendrin[34,35] — shares homology with the calmodulin-binding domain of the yeast γ-tubulin-binding protein Spc110, and so seems to be the vertebrate homologue. Pericentrin B has characteristics that are distinct from pericentrin: it is present in protein complexes of different *S* VALUES and isoform-specific antibodies do not seem to inhibit aster formation in *Spisula solidissima* extracts, whereas pericentrin antibodies inhibit asters in *Xenopus* extracts[6,36]. Future studies will be required to understand the role of different pericentrin family members and other molecules in the recruitment of γ-tubulin complexes to centrosomes.

Polo and aurora kinases are localized to centrosomes and are important for centrosome integrity and separation, respectively[37]. Polo mutants in *Drosophila* disrupt the organization of both mitotic spindles and centrosome-associated γ-tubulin[38,39]. Extracts of *polo*-mutant embryos cannot restore the ability of salt-stripped centrosomes to nucleate microtubule asters. This can be rescued by addition of phosphorylated Asp or active Polo kinase. Polo and Asp co-fractionate in gradients to apposition, consistent with a large complex (25–38 S)[39], and they co-immunoprecipitate with one another and with γ-tubulin[40]. Moreover, Polo phosphorylates Asp *in vitro*[39], indicating a possible mechanism for mitotic centrosome assembly in which Polo-activated Asp binds to, and anchors, γ-tubulin complexes to centrosomes.

Microtubule anchoring. It seems likely that microtubule nucleation and microtubule anchoring are two separate activities, mediated by different classes of molecules. Data supporting a separation of these activities come from the analysis of unique microtubule-anchoring sites at the apical domains of epithelial cells[41] (FIG. 2). These sites function as anchoring sites for the minus ends of hundreds of microtubules, but they lack the components that are usually associated with microtubule nucleation such as γ-tubulin and pericentrin. So far, only two proteins have been localized to these specialized microtubule-anchoring sites — ninein[42] and centriolin[43]. These proteins are also associated with the subdistal appendages of the maternal centriole, which also act as microtubule-anchoring sites and also lack microtubule-nucleating proteins[44,45].

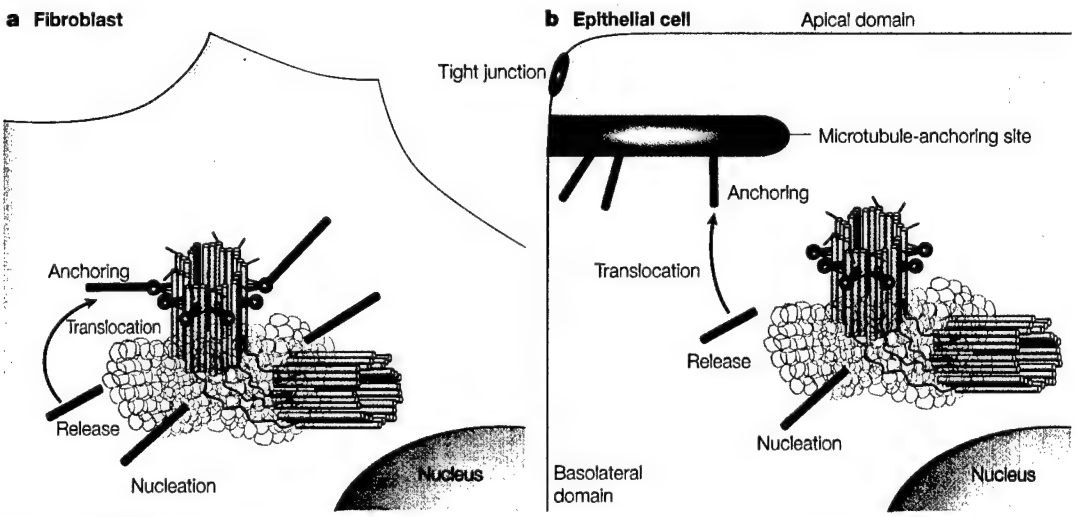

Figure 2 | **Model for microtubule nucleation, release and anchoring at centrosomes and in epithelial cells.** In this model, microtubules are nucleated in the pericentriolar material, released from their nucleating sites and translocated to sites of anchoring. **a** | Anchoring at the centrosome occurs at the subdistal appendages whereas in **b** | epithelial cells, it occurs at apical domains distinct from centrosomes. Despite the apparent differences in anchoring sites, both contain unique proteins (ninein and centriolin) and seem to lack components involved in microtubule nucleation (γ-tubulin and pericentrin). Dynactin, a dynein-interacting complex, has also been implicated in centrosomal microtubule anchoring[127].

KINETOCHORE
A structure that connects each chromatid to the spindle microtubules, which shorten as pairs of chromatids are separated to opposite poles.

During mitosis, a different type of minus-end microtubule anchoring is probably required. Mitotic microtubules are attached at their plus ends to specialized sites on chromosomes called KINETOCHORES (see the review by Kitagawa and Hieter on page 678 of this issue), and anchored at their minus ends to centrosomes or spindle poles. However, these interactions are not static — α- and β-tubulin subunits are continuously added at the kinetochore end and are lost from the end that is embedded in the spindle pole. This results in a continuous flux of the microtubule, with no net movement of chromosomes or spindle poles[46]. To maintain this dynamic association requires molecules that can remain attached to moving microtubules, such as spindle-pole-associated motor proteins.

Microtubule release. Microtubule release from centrosomes has been observed in somatic cells[47]. One candidate for this activity is the centrosome-associated protein katanin, which has been shown to sever microtubules *in vitro*[48] and *in vivo*[49]. Other candidates include members of the Kin1 subfamily of kinesins (XKCM1 and XKIF2) that destabilize *in-vitro*-assembled microtubules equally at minus and plus ends[50]. Further studies should determine whether katanin, members of the Kin1 family, or other proteins induce microtubule release in interphase and mitotic cells[47].

A hypothetical model that summarizes the microtubule nucleation, severing and anchoring functions of the centrosome is shown in FIG. 3. Microtubules nucleated by the pericentriolar material of centrosomes are released[47,49], perhaps through the activity of microtubule-severing proteins. They are then translocated to apical domains in specialized epithelial cells or to subdistal appendages of centrioles where they are anchored. The mechanisms by which microtubules are severed, translocated and anchored at sites distinct from those that mediate microtubule nucleation are important unresolved issues.

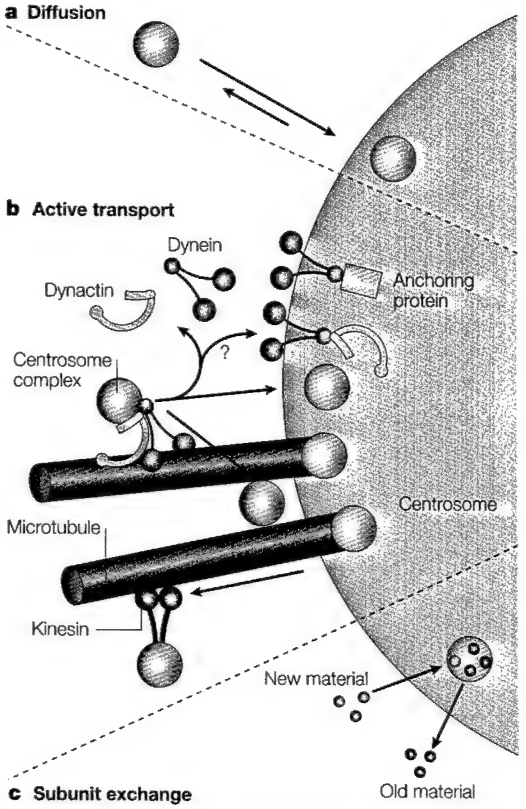

Figure 3 | **Models for centrosome protein assembly.** **a** | Centrosome complexes diffuse to centrosomes and assemble in a microtubule- and dynein-independent manner. **b** | Centrosome complexes, in the form of large particles, assemble using microtubules and cytoplasmic dynein motors. Particles are delivered to centrosomes or to areas between microtubules near the centrosome. Dynein motors may be anchored at centrosomes by dynactin or other anchoring proteins, or released after cargo delivery. **c** | Subunits exchange with those at the centrosome in either **a** or **b**. This could occur continuously or in a cell-cycle-dependent manner.

Centrosome dynamics

Many centrosome proteins undergo cell-cycle-regulated assembly onto centrosomes from cytoplasmic pools as protein complexes or larger protein particles (FIG. 3; see REF. 20 for a review). Cytoplasmic dynein has been shown to mediate the assembly of centrosome/spindle pole proteins in both vertebrate cell systems and embryonic systems[20]. Dynein interacts with many centrosome/spindle pole proteins[51–53] and mediates their interaction with microtubules[54,55]. Moreover, these proteins undergo centripetal, microtubule-dependent movements that end at the centrosome, and their accumulation at centrosomes depends on dynein function[55–57]. Localization of centrosome proteins could occur by retrograde, dynein-dependent transport along microtubule tracks, in a process that is similar to intraflagellar transport (BOX 1).

Microtubule and dynein-independent mechanisms for assembly of centrosome proteins also exist, both in embryonic systems[17,21,55,58,59] and in vertebrate cells[60]. It is possible that dynein-dependent and dynein-independent mechanisms represent redundant pathways for centrosome assembly, with one predominating in certain biological systems or during different cell-cycle stages.

Centrioles are remarkably dynamic. In cells that express centrin fused to green fluorescent protein (GFP), both mother and daughter centrioles can undergo marked movements and move independently of one another[61,62]. Moreover, during the final stages of cell division (cytokinesis), the mother centriole moves across the entire cell to a position near the site of cell cleavage (FIG. 4). Some of these mitotic movements are microtubule-dependent and might be mediated through an interaction between centriole-associated microtubules and regions of the plasma membrane near the site of cell cleavage.

Centrosomes and mitotic spindle assembly?

The role of the centrosome in spindle assembly and function was suggested as far back as the late 1800s, when centrosomes were first identified. It seemed logical that

Box 1 | Intraflagellar transport and centrosome assembly

The process of centrosome protein assembly shares features with intraflagellar transport. In intraflagellar transport, large multiprotein particles or 'rafts' shuttle between the basal bodies and flagellar tips. The rafts seem to carry pre-assembled protein cargoes to the tips of cilia and flagella and return to the basal bodies to reload[126]. Kinesin and cytoplasmic dynein are involved in the transport to and from the flagellar tip, respectively. The recruitment of centrosome proteins might use a similar transport system with net movement towards centrosomes. Identification of protein carriers analogous to rafts will be required to substantiate this idea.

ANEUPLOIDY
Presence of extra copies, or no copies, of some chromosomes.

centrosomes have a role in spindle assembly through their microtubule-nucleating and organizing functions and their association with the spindle poles. However, higher plants and many developmental systems lack canonical centrosomes but can still organize normal spindles and undergo cell division[63–65]. Moreover, recent data indicate that centrosomes are not essential for spindle assembly in mammalian cells[66,67], suggesting that most — if not all — cells can assemble spindles by non-centrosomal pathways.

If centrosomes are not essential for spindle assembly then what is their role in this process? When present, centrosomes act dominantly to organize spindle poles[68]. Centrosome-mediated spindle assembly could provide a redundant pathway to ensure high fidelity of chromosome segregation. Alternatively, it could ensure that centrosomes are inherited during each cell division so that they can complete other essential cellular functions (see below).

Centrosome dominance in spindle-pole formation has important implications in human cancer. When present in more than two copies, centrosomes contribute to the assembly of multipolar spindles that can missegregate chromosomes, creating ANEUPLOIDY. So, although the role of centrosomes in bipolar spindle assembly is unclear, their dominant role in spindle-pole formation might contribute to the genetic instability that is observed in tumour cells with supernumerary centrosomes.

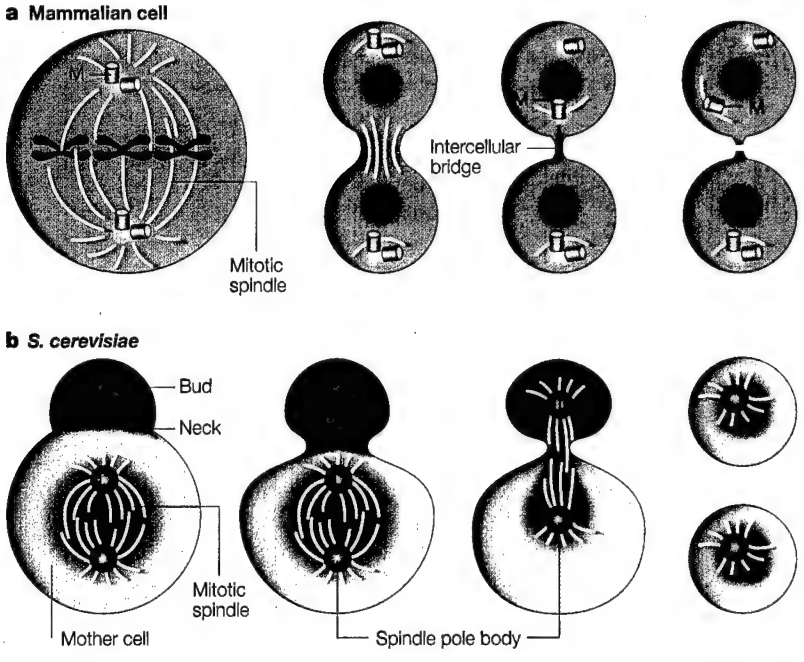

Figure 4 | **Centrosome and spindle pole body positioning during cytokinesis. a** | During cytokinesis in a mammalian cell, the maternal (M) centriole moves to the intercellular bridge. Subsequent to this movement, the microtubules within the bridge depolymerize and the bridge constricts. Movement of the centriole away from the bridge seems to release the arrest in cytokinesis and the cell cleaves. **b** | The molecular details of a parallel pathway in budding yeast are better defined. Tem1 on the spindle pole body nearest the bud is modified from a GDP (D)-bound form to a GTP (T)-bound form when the spindle pole body enters the bud and meets the GTP-exchange factor Lte1 (dark orange). This event, together with destabilization of microtubules or loss of microtubule attachment to the bud neck, stimulates mitotic exit and cell cleavage.

Positioning of the mitotic spindle

Positioning of the spindle within cells is important for several fundamental processes, including accurate segregation of chromosomes, asymmetric distribution of cell-fate determinants during development, normal and asymmetric cell divisions and defining the plane of cytokinesis. Centrosomes and associated astral microtubules seem to be important for spindle positioning in mammalian and yeast cells[69–71]. A common feature of these systems is the interaction of molecules at the plus ends of centrosome-associated astral microtubules, with cytoplasmic dynein located in the cell cortex[72]. Forces generated outside the central spindle by dynein contribute, at least in part, to the driving force for spindle positioning[73,74]. In mammalian cells that lack centrosomes, no astral microtubules are produced and spindles become mispositioned[75], causing subsequent problems during cytokinesis. Other cytoskeletal elements and motors function during spindle positioning[76–78], and recent studies indicate that non-centrosomal mechanisms for spindle positioning also exist[65,79,80].

Cytokinesis/checkpoint activation

The role of the mitotic spindle in defining the site of cell cleavage during cytokinesis has been known for some time[81]. Recent studies with vertebrate cells link centrosome activity to the completion of cytokinesis and progression from G1 to S phase of the cell cycle. If centrosomes are eliminated from interphase cells by removal with a microneedle[66] or from mitotic cells by laser ablation[67], mitotic cells can still assemble mitotic spindles but roughly half fail to complete cytokinesis. Cells appear to remain attached by intercellular bridges or abort cytokinesis, forming binucleate cells.

In another study in which centrioles were labelled with centrin coupled to GFP[61], the final events of cell cleavage correlated with movement of the maternal centriole to the intercellular bridge that connects dividing cells, and this localization correlated with bridge narrowing and microtubule depolymerization within the bridge. Movement of the centriole away from the bridge occurred before cell cleavage. Moreover, conditions that adversely affected centriole movement, such as changes in cell adherence or confluency, delayed cytokinesis (FIG. 4). These studies indicate that centrosomes might be involved in the activation of the final stages of cytokinesis or in the release

Box 2 | **Chromosome and centrosome duplication**

The unique ability of centrosomes to duplicate once every cell cycle is shared by only one other cellular organelle — chromosomes. The mechanism of chromosomal DNA replication is relatively straightforward — pairing of nucleotide subunits with their complementary partners on a DNA template. But centrosome duplication seems to be much more complicated.

Despite these differences, there are remarkable similarities between the two processes. First, both are initiated at roughly the same time in the cell cycle, the G1 to S transition. They are regulated by the same protein complexes containing Cdk2 and cyclin A/E. They both seem to require proteolysis of cohesive material to separate their subunits (sister chromatids and parental centrioles). Finally, replication of both DNA and centrosomes is semiconservative, with each newly replicated unit containing both new and old subunits.

One important difference between the two processes seems to be the time that it will take to understand them fully. Whereas the mechanism of DNA replication was suggested immediately upon discovery of a structure consistent with a templating mechanism, the centrosome duplication process seems to be much more complex, and is likely to keep many investigators busy for some time.

of cells from a CHECKPOINT that monitors the completion of mitosis.

Results from budding yeast are consistent with release from a checkpoint. Recent studies show that yeast cells remain in cytokinesis until the spindle pole body moves into the nascent daughter cell (bud), bringing into contact the GDP-bound form of Tem1 at the spindle pole body and a GUANINE-NUCLEOTIDE EXCHANGE FACTOR, Lte1, in the bud, thus producing the activated Tem1–GTP. These events, together with microtubule depolymerization at the bud junction (neck) and loss of microtubule contact with the neck[82], trigger cytokinesis and the exit from mitosis[83–86] (FIG. 4). So, some of the events in this pathway seem to be similar between yeast and vertebrates[87,88].

The G1 to S transition

In the absence of centrosomes, somatic cells arrest in G1, and do not initiate DNA replication[66,75]. It is possible that the cells do not divide completely, but that they remain attached by thin intercellular bridges or divide by crawling apart — a process that is not likely to occur in normal tissues, owing to the crowded environment. In either case, these cells might activate a checkpoint that monitors aberrant centrosome numbers or perhaps the presence of excess DNA (binucleate cells)[89]. Results from fission yeast[90,91], in which cytokinesis failure triggers a checkpoint that inhibits progression of the nuclear cell cycle (albeit at a later stage in the cell cycle; G2) are consistent with this model. Alternatively, centrosomes might be required to activate DNA replication — perhaps through the recruitment or concentration of molecules that are essential for the initiation of DNA synthesis[92,93]. It is still unclear whether centrosomes directly mediate these events, or whether centrosome defects trigger checkpoints that monitor the completion of mitosis. Regardless of the mechanism, one consequence of having a centrosome requirement for cell-cycle progression is to ensure that dividing animal cells receive the appropriate number of functional centrosomes.

CHECKPOINT
A point where the cell division cycle can be halted until conditions are suitable for the cell to proceed to the next stage.

GUANINE-NUCLEOTIDE EXCHANGE FACTOR
A protein that facilitates the exchange of GDP (guanine diphosphate) for GTP (guanine triphosphate) in the nucleotide-binding pocket of a GTP-binding protein.

Actin polymerization

Centrosomes might also have a role in coordinating actin polymerization during pseudocleavage in *Drosophila* embryos — an event similar to cytokinesis. This is not surprising, given the role of the spindle poles/centrosomes in defining the site of cytokinesis[81]. The unexpected observation in this study is that centrosome-mediated actin polymerization seems to be independent of microtubules[94]. This suggests that actin polymerization is controlled by a signal that diffuses from centrosomes or that is carried from centrosomes by a microtubule-independent mechanism. This observation raises the possibility that centrosomes act as diffusion centres for signalling molecules, thus allowing them to influence processes elsewhere in the cell. Centrosomal gradients of activities could also be established in this way and could provide different cytoplasmic environments that activate or inhibit cellular processes in much the same way that protein gradients establish patterning in *Drosophila* embryos[95].

Monitoring DNA damage

Another role for centrosomes might be in monitoring DNA damage. During early embryogenesis of *Drosophila*, mutations in the DNA-replication checkpoint lead to failures in chromosome segregation. A recent study shows that centrosomes in *Drosophila* embryos respond to mutations in the DNA-replication checkpoint by inactivating centrosomes in mitosis[96]. Identical results are obtained when normal embryos are treated with DNA-damaging agents or DNA-replication inhibitors. In all cases, mitotic centrosomes lose components of the γ-tubulin ring complex and the ability to nucleate astral microtubules. Inactivation of centrosomes might be part of a damage-control system that prevents chromosome segregation if the checkpoint that monitors DNA replication or damage fails.

Centrosome duplication

Much like chromosomes, centrosomes duplicate precisely once every cell cycle (BOX 2). The fidelity and timing of centrosome duplication is essential for ensuring that this process is effectively coupled to other events, such as cell-cycle progression and DNA replication[97]. Uncoupling of these events can lead to excess centrosomes that organize multipolar spindles or single centrosomes with associated monopolar spindles. Recent studies from several laboratories have provided important insights into the mechanism and regulation of centrosome duplication.

The centrosome cycle. A typical somatic cell in the G1 phase of the cell cycle contains one centrosome with two centrioles. The first visible sign of centrosome duplication is splitting of the two centrioles at the G1/S transition (FIG. 5). At this early stage in the duplication process, the older mother centriole (FIG. 1) and its associated pericentriolar material can be distinguished from the younger daughter centriole. It has appendages at the end farthest from the daughter and a subset of integral centrosome proteins that includes cenexin[98],

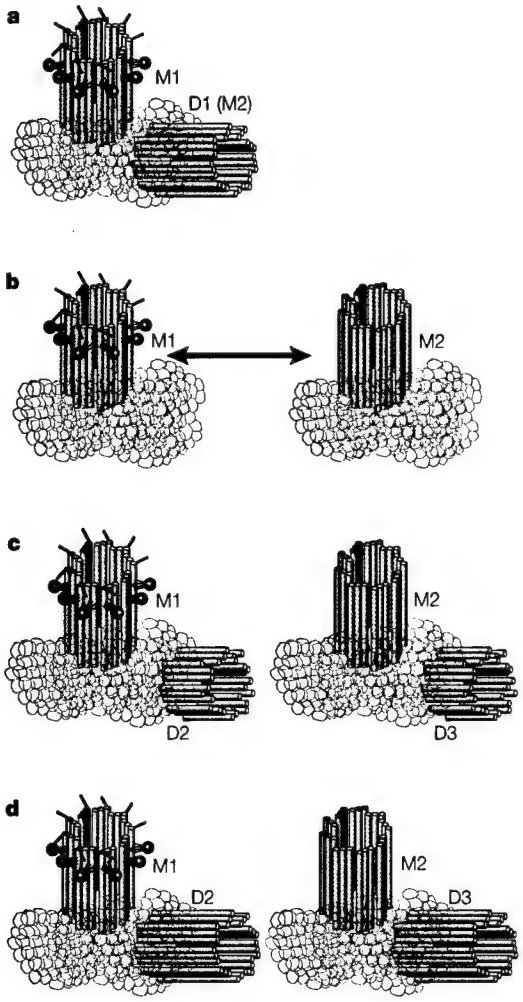

Figure 5 | **Centrosome duplication. a** | In early G1, a centrosome has daughter (D) and mother (M) centrioles. **b** | The centrioles move apart and **c** | each nucleates the formation of a new centriole during G1 to S. **d** | By mitosis, the new centrioles have reached their full length and the two mature centrosomes, each with two centrioles, are segregated into the daughter cells upon cell division.

ninein[42], ε-tubulin[99], centrin[100] and centriolin[43]. During S phase, new centrioles arise from the sides of the original centrioles and elongate through G2 to achieve their full length by late G2 or M phase. Although it would seem simpler and more efficient for new centrioles to grow from the pre-existing templates provided by centriole ends, growth occurs in material closely apposed to the centriole and probably requires γ-tubulin[101]. To complicate matters, basal bodies and centrioles can arise from amorphous cytoplasmic aggregates that have no discernible templates[102,103].

During the duplication process, centriolar microtubules elongate and many centriolar and pericentriolar components accumulate[104]. In G2/early mitosis, the duplicated centrosomes separate in a two-part process. First, material interconnecting the centrosomes seems to be severed by the activity of a centrosomal kinase, NEK2, acting on a putative centrosomal substrate (cNAP1)[105,106]. In a second step, the duplicated centrosomes move apart by the concerted action of molecular motors on microtubules between the two centrosomes[107], and the separated centrosomes subsequently participate in the organization of mitotic spindles.

Regulation. Recent studies indicate that many regulatory pathways might control centrosome duplication. Many of these regulatory proteins seem to interact with centrosomes, although it is still unclear whether they exert their influence locally or more globally. Cell-cycle progression from G1 to S phase requires the activity of protein complexes containing the centrosome-associated cell-cycle kinase Cdk2 and cyclin E. In *Xenopus* embryos and egg extracts, inhibitors of Cdk2 block centrosome duplication[92,93], apparently at one of the earliest stages — the splitting of centrioles[93]. Cdk2 is also required for centrosome duplication in mammalian cells[108,109]. However, cyclin A seems to predominate in mammalian cell systems[109] whereas cyclin E is more active in *Xenopus*[93]. Differential binding of cyclins A or E in the same organism could also mediate Cdk activity at different times during the duplication process.

One downstream phosphorylation target of Cdk2–cyclin E is the mouse ORTHOLOGUE of the yeast Mps1 protein kinase. Mps1 is a Cdk2 substrate that seems to regulate centrosome duplication jointly with Cdk2 (REF. 110). Another target of Cdk2–cyclin E is nucleophosmin[111]. This protein is localized to centrosomes before duplication (late M to G1), and is released from centrosomes during duplication after phosphorylation by Cdk2–cyclin E. Inhibition of nucleophosmin in mammalian cells by antibodies or by overexpression of a mutant protein that lacks the Cdk2–cyclin E phosphorylation site inhibits centrosome duplication at an early stage[111,112]. These data indicate that nucleophosmin might regulate centrosome duplication by preventing centriole splitting until it is released from centrosomes or degraded. It is possible that nucleophosmin is ultimately regulated by p53–p21$^{Waf1/Cip}$, indicating a potential mechanism by which centrosome duplication might be abnormal in tumours[113].

Consistent with the degradation model is the observation that the ubiquitin-mediated proteolysis pathway is required for centrosome duplication. Inhibition of several components of the SCF COMPLEX and the PROTEASOME in *Xenopus* extracts and embryos inhibited centrosome duplication and prevented centriole splitting, supporting the idea that proteolysis is required to sever connections between centrioles early in the centrosome duplication process[114]. Other studies have shown that mutations or knockouts of SCF components in mice and *Drosophila* result in excess centrosomes, suggesting that the proteolysis machinery limits centrosome duplication[115,116]. Additional studies will be required to define the precise role of proteolysis in duplication and to determine whether the molecular targets of proteolysis are at the centrosome or elsewhere.

ORTHOLOGUE
Homologous genes in different species, the lineages of which derive from a common ancestral gene without gene duplication or horizontal transmission.

SCF COMPLEX
A multisubunit ubiquitin ligase that contains Skp1, a member of the cullin family (Cul1), and an F-box-containing protein (Skp2), as well as a RING-finger-containing protein (Roc1/Rbx1).

PROTEASOME
Large multisubunit protease complex that selectively degrades intracellular proteins. Targeting to proteasomes most often occurs through attachment of multi-ubiquitin tags.

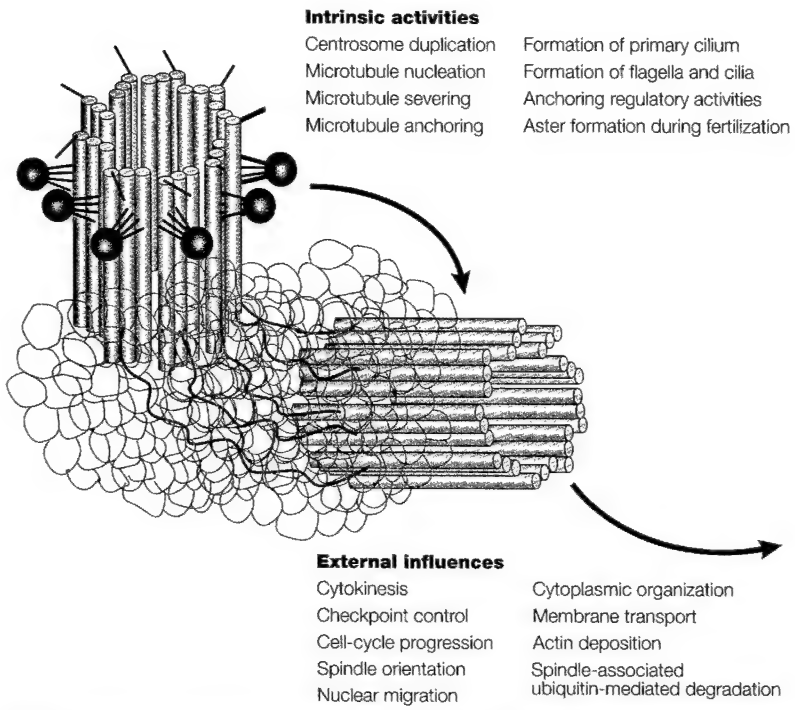

Figure 6 | **Intrinsic activities and external influences of centrosomes.** The current status of centrosome activities that affect centrosomes (intrinsic activities) and those that influence other cellular sites or functions (external influences) is shown.

An apparently later step in the centrosome-duplication pathway was recently discovered. The *Caenorhabditis elegans* ZYG-1 protein is a novel kinase that is presumably required for the formation of new centrioles. *zyg-1* mutants form monopolar spindles with single centrosomes containing only one centriole[117]. However, cell-cycle progression is generally unaffected in these mutants, and the ability of centrioles to split is apparently not impaired. ZYG-1 localizes to centrosomes from anaphase to the beginning of G1 — the time when *C. elegans* embryos, like many other embryonic systems, initiate centrosome duplication. These data indicate that ZYG-1 acts at a stage of the duplication process after centriole splitting, most likely during the formation of nascent centrioles. It will be interesting to determine whether the kinase is part of a novel regulatory pathway or whether it directly phosphorylates centrosome proteins that initiate new centriole formation. Also of interest is whether it interacts functionally with other pathways that control centrosome duplication such as Cdk2–cyclin E, SCF and Mps1.

MALD TOF
A method designed to determine peptide mass maps of very small amounts of enzymatically digested proteins with a very high degree of accuracy. Masses are determined by measuring peptides that are ionized in a vacuum.

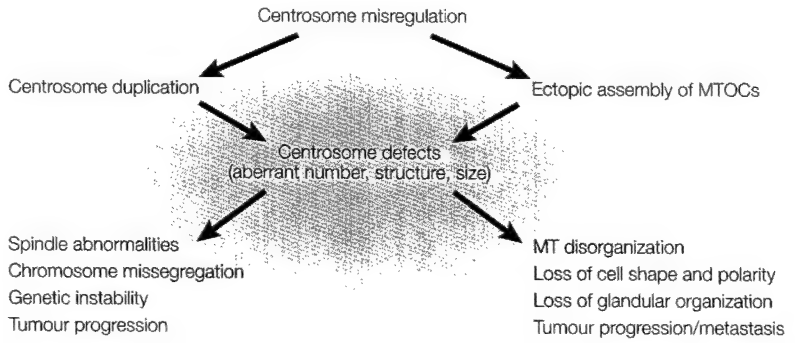

Figure 7 | **Two pathways for generating centrosome defects that could lead to genetic instability and loss of cell polarity in cancer.** One pathway affects the centrosome-duplication cycle (left) and the other affects centrosome assembly and integrity (right). Regardless of the pathway, centrosome defects have the potential to generate genetic instability and loss of cell polarity and shape, and thus contribute to tumorigenesis. MT, microtubule; MTOC, microtubule-organizing centre.

Anchoring of regulatory activities

It is now widely accepted that centrosomes and spindle poles act as anchoring sites for molecular regulators of cellular functions, distinct from functions typically ascribed to centrosomes. Molecules that associate with centrosomes are involved in a diverse set of cellular functions, including cell-cycle progression, checkpoint control, centrosome and spindle function and ubiquitin-mediated degradation (FIG. 6). However, little is known about the significance of their centrosome localization. In *Drosophila* embryos, cyclin B localizes to centrosomes during mitosis and is degraded as cells transit from metaphase to anaphase. Degradation proceeds from the centrosome up the spindle to the chromosomes, and is retarded if the centrosome is detached from the spindle pole[118,119]. So it seems that the degradation of cyclins — and perhaps other proteins — is a solid-state phenomenon in that it is confined to a distinct cellular location.

A family of centrosomal proteins has recently been identified that binds cAMP-dependent protein kinase A (PKA) (for reviews see REFS 120,121). These A-kinase-anchoring proteins (AKAPs) are found at many cellular sites, where they function as molecular scaffolds not only for PKA, but also for other kinases (protein kinase C, for example), phosphatases and perhaps other regulatory molecules. By interacting with regulatory molecules, AKAPs integrate diverse signalling pathways that regulate the phosphorylation of specific cellular substrates and effectors. The role of centrosome-associated AKAPs, as well as the rapidly growing list of other regulatory molecules is still largely unknown.

Future directions

During the past five to ten years, there has been a change in the perception of the role of centrosomes. Their role in spindle assembly is in question as spindles form in the absence of centrosomes. However, centrosomes have a dominant role in spindle assembly when present. New roles for centrosomes are emerging. They seem to be involved in the completion of cytokinesis and in cell-cycle progression. This new glimpse into the complexity of these organelles suggests that we are only beginning to uncover their true functions.

Our understanding of centrosome composition is still limited. A full appreciation of how centrosomes contribute to cellular function will require isolation and characterization of the estimated hundreds of centrosome-associated molecules. The use of matrix-assisted-laser-desorbtion time-of-flight (MALDI TOF)

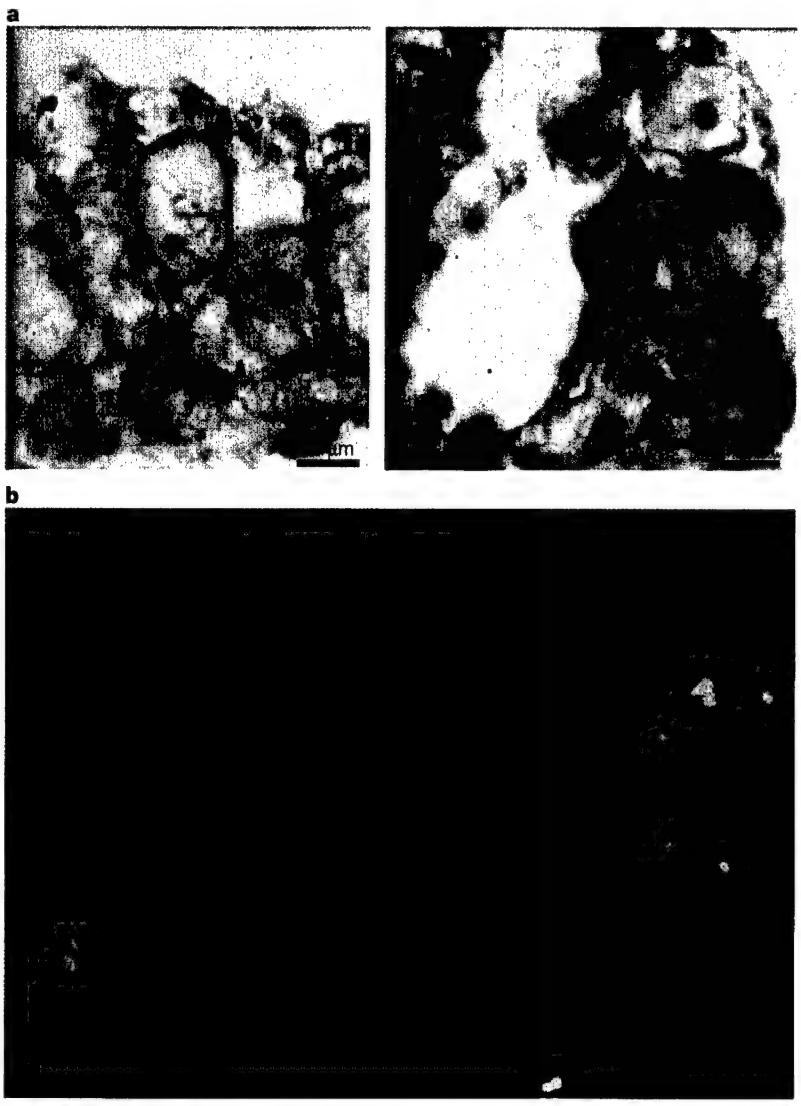

Figure 8 | **Abnormal centrosomes in a human tumour and in a human tumour cell line.** **a** | A human tumour (paraffin section), stained by immunohistochemistry with antibodies to pericentrin (brown) shows normal centrosomes (left), whereas centrosomes in adjacent tumour cells (right, same magnification) show gross abnormalities (larger diameter, elongated forms, supernumerary and acentriolar forms). **b** | Two human breast tumour cells (right) and a non-tumour cell (left bottom) treated with nocodazole to depolymerize microtubules, then washed free of the drug to allow microtubule regrowth from centrosomes. Microtubules, green; centrosomes (pericentrin), red or yellow; nuclei, blue. Note the large number and heterogeneity in size and shape of pericentrin-staining structures in tumour cells, many of which lack centrioles but contain nucleated microtubules and contribute to dysfunctional spindles (not shown). The non-tumour cell has the typical single centrosome and single focus of microtubules. Reproduced with permission from REF. 128 © (1998) American Association for Cancer Research.

mass spectrometry will facilitate analysis of centrosome fractions and provide a framework for piecing together centrosome molecules, in much the same way that this technology has facilitated the functional characterization and molecular organization of the yeast spindle pole body[4].

An under-explored question in centrosome biology is how centrosome-anchored regulatory proteins and messenger RNAs[122] control cellular function. Many molecules localized to centrosomes are part of signal-transduction pathways[120] and others are known to regulate various cellular activities other than centrosome function (reviewed in REFS 87,123). However, the functional significance of the centrosome association of these molecules and how centrosomes communicate with the outside of the cell remain to be determined.

Another area of centrosome biology that is likely to yield exciting results in the near future is the role of centrosomes in generating genetic instability (FIG. 7). As centrosomes act dominantly when present in somatic cells[68], they contribute to spindle abnormalities, chromosome missegregation and genetic instability — either alone or in combination with alterations in other cellular pathways (such as apoptosis, cell-cycle progression, cell-cycle checkpoints and cell-growth regulation) that have been implicated in the genetic instability that is observed in many cancers. Increased centrosome numbers and centrosome defects have been observed in most aggressive tumours (FIG. 8) and in some low-grade tumours and pre-cancerous lesions[124,125]. These centrosome abnormalities could be generated by misregulation of centrosome duplication, or by ectopic assembly of microtubule-nucleating material in the absence of centrioles. Because both centrosomes and acentriolar MTOCs organize dysfunctional mitotic spindles, both have the potential to contribute to genetic instability during tumorigenesis. Also in support of a role for centrosomes in cancer is the observation that tumour-like features can be induced *in vitro* by modifying the levels of centrosome proteins and centrosome-associated kinases linked to tumorigenesis[124]. However, a direct link between centrosome defects and cancer has not yet been established.

The next five to ten years in centrosome research promise to be very exciting. The emerging view of the centrosome is one of a command centre that receives, integrates and transmits signals that regulate cellular activities[87]. In this way, centrosomes may process signals from the external environment through associated microtubules and primary cilia[10], through association of centrioles with the plasma membrane[61] or through signal-transduction pathways[120]. These signals could be transmitted to other cellular sites or between adjacent cells in tissues. A better understanding of centrosomes beyond that of microtubule organizers will provide important insights into fundamental issues about cell regulation, and will probably have an important impact on the aetiology and control of diseases such as cancer.

Links

DATABASE LINKS γ-tubulin | α-tubulin | β-tubulin | Spc97 | Spc98 | Tub4 | Spc72 | Spc110 | Asp | GCP2 | GCP3 | pericentrin | pericentrin B | polo | aurora | ninein | katanin | XKCM1 | XKIF2 | cytoplasmic dynein | centrin | Tem1 | Lte1 | ε-tubulin | NEK2 | cNAP1 | Cdk2 | cyclin E | cyclin A | Mps1 | nucleophosmin | p53 | p21 | ZYG-1 | cyclin B

FURTHER INFORMATION movie of pericentrin | movie of PCM-1

1. Wheatley, D. N. *The Centriole* (Elsevier Biomedical Press, Amsterdam, 1982).
2. Francis, S. E. & Davis, T. N. The spindle pole body of *Saccharomyces cerevisiae*: architecture and assembly of the core components. *Curr. Top. Dev. Biol.* **49**, 105–132 (2000).
3. Schiebel, E. γ-tubulin complexes: binding to the centrosome, regulation and microtubule nucleation. *Curr. Opin. Cell Biol.* **12**, 113–118 (2000).
4. Wigge, P. *et al.* Analysis of the *Saccharomyces* spindle pole by matrix-assisted laser desorbtion/ionization (MALDI) mass spectrometry. *J. Cell Biol.* **141**, 967–977 (1998).
5. Gould, R. R. & Borisy, G. G. The pericentriolar material in Chinese hamster ovary cells nucleates microtubule formation. *J. Cell Biol.* **73**, 601–615 (1990).
6. Dictenberg, J. *et al.* Pericentrin and γ-tubulin form a protein complex and are organized into a novel lattice at the centrosome. *J. Cell Biol.* **141**, 163–174 (1998).
Identifies pericentrin as a protein that interacts with the γ-tubulin ring complex and co-localizes with γ-tubulin at the centrosome (also see reference 34).
7. Vogel, J. M., Stearns, T., Rieder, C. L. & Palazzo, R. E. Centrosomes isolated from *Spistula solidissima* oocytes contain rings and an unusual stoichiometric ratio of α/β-tubulin. *J. Cell Biol.* **137**, 193–202 (1997).
8. Moritz, M., Braunfeld, M. B., Sedat, J. W., Alberts, B. & Agard, D. A. Microtubule nucleation by γ-tubulin-containing rings in the centrosome. *Nature* **378**, 638–640 (1995).
9. Wheatley, D. N., Wang, A. M. & Strugnell, G. E. Expression of primary cilia in mammalian cells. *Cell Biol. Int.* **20**, 73–81 (1996).
10. Pazour, G. J. *et al. Chlamydomonas IFT88* and its mouse homologue, polycystic kidney disease gene *tg737*, are required for assembly of cilia and flagella. *J. Cell Biol.* **151**, 709–718 (2000).
Demonstration that a centriole protein required for formation of cilia and flagella is involved in polycystic kidney disease.
11. Hewitson, L., Simerly, C. & Schatten, G. Cytoskeletal aspects of assisted fertilization. *Semin. Reprod. Med.* **18**, 151–159 (2000).
12. Manandhar, G., Simerly, C. & Schatten, G. Highly degenerated distal centrioles in rhesus and human spermatozoa. *Hum. Reprod.* **15**, 256–263 (2000).
13. Bobinnec, Y. *et al.* Centriole disassembly *in vivo* and its effect on centrosome structure and function in vertebrate cells. *J. Cell Biol.* **143**, 1575–1589 (1998).
Shows that disruption of centrioles causes dispersion of PCM components, indicating a role for centrioles in centrosome organization.
14. Evans, L., Mitchison, T. & Kirschner, M. Influence of the centrosome on the structure of nucleated microtubules. *J. Cell Biol.* **100**, 1185–1191 (1985).
15. Oakley, B. R., Oakley, C. E., Yoon, Y. & Jung, M. K. γ-tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Nature* **338**, 662–664 (1990).
16. Zheng, Y., Wong, M. L., Alberts, B. & Mitchison, T. Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. *Nature* **378**, 578–583 (1995).
17. Stearns, T. & Kirschner, M. Reconstitution of centrosome assembly, role of γ-tubulin. *Cell* **76**, 623–637 (1994).
18. Wiese, C. & Zheng, Y. A new function for the γ-tubulin ring complex as a microtubule minus-end cap. *Nature Cell Biol.* **2**, 358–364 (2000).
19. Zimmerman, W., Sparks, C. A. & Doxsey, S. J. Amorphous no longer: the centrosome comes into focus. *Curr. Opin. Cell Biol.* **11**, 122–128 (1999).
20. Zimmerman, W. & Doxsey, S. J. Construction of centrosomes and spindle poles by molecular motor-driven assembly of protein particles. *Traffic* **1**, 927–934 (2000).
21. Moritz, M., Zheng, Y., Alberts, B. M. & Oegema, K. Recruitment of the γ-tubulin ring complex to *Drosophila* salt-stripped centrosome scaffolds. *J. Cell Biol.* **142**, 775–786 (1998).
22. Oegema, K. *et al.* Characterization of two related *Drosophila* γ-tubulin complexes that differ in their ability to nucleate microtubules. *J. Cell Biol.* **144**, 721–733 (1999).
Identification of two γ-tubulin complexes of different sizes in *Drosophila* embryos.
23. Leguy, R., Melki, R., Pantaloni, D. & Carlier, M. F. Monomeric γ-tubulin nucleates microtubules. *J. Biol. Chem.* **275**, 21975–21980 (2000).
24. Murphy, S. M., Urbani, L. & Stearns, T. The mammalian γ-tubulin complex contains homologues of the yeast spindle pole body components spc97p and spc98p. *J. Cell Biol.* **141**, 663–674 (1998).
25. Gunawardane, R. N., Lizarraga, S. B., Wiese, C., Wilde, A. & Zheng, Y. γ-tubulin complexes and their role in microtubule nucleation. *Curr. Top. Dev. Biol.* **49**, 55–73 (2000).
26. Keating, T. J. & Borisy, G. G. Immunostructural evidence for the template mechanism of microtubule nucleation. *Nature Cell Biol.* **2**, 352–357 (2000).
27. Moritz, M., Braunfeld, M. B., Guenebaut, V., Heuser, J. & Agard, D. A. Structure of the γ-tubulin ring complex: a template for microtubule nucleation. *Nature Cell Biol.* **2**, 365–370 (2000).
28. Erickson, H. P. & Stoffler, D. Protofilaments and rings, two conformations of the tubulin family conserved from bacterial FtsZ to α/β- and γ-tubulin. *J. Cell Biol.* **135**, 5–8 (1996).
29. Erickson, H. P. γ-tubulin nucleation: template or protofilament? *Nature Cell Biol.* **2**, E93–E96 (2000).
30. Knop, M. & Schiebel, E. Spc98p and Spc97p of the yeast γ-tubulin complex mediate binding to the spindle pole body via their interaction with Spc110p. *EMBO J.* **16**, 6985–6995 (1997).
31. Knop, M. & Schiebel, E. Receptors determine the cellular localization of a γ-tubulin complex and thereby the site of microtubule formation. *EMBO J.* **17**, 3952–3967 (1998).
32. do Carmo Avides, M. & Glover, D. M. Abnormal spindle protein, ASP, and the integrity of mitotic centrosomal microtubule organizing centers. *Science* **283**, 1733–1735 (1999).
33. Tassin, A. M., Celati, C., Paintrand, M. & Bornens, M. Identification of an Spc110p-related protein in vertebrates. *J. Cell Sci.* **110**, 2533–2545 (1997).
34. Flory, M. R., Moser, M. J., Monnat, R. J. Jr & Davis, T. N. Identification of a human centrosomal calmodulin-binding protein that shares homology with pericentrin. *Proc. Natl Acad. Sci. USA* **97**, 5919–5923 (2000).
Shows that an apparent isoform of human pericentrin, pericentrinB/kendrin has a calmodulin-binding domain that is homologous to the yeast protein Spc110, which binds γ-tubulin complexes at the spindle pole body (also see reference 6).
35. Li, Q. *et al.* Kendrin/pericentrin-B, a centrosome protein with homology to pericentrin that complexes with PCM-1. *J. Cell Sci.* **114**, 797–809 (2001).
36. Doxsey, S. J., Stein, P., Evans, L., Calarco, P. & Kirschner, M. Pericentrin, a highly conserved protein of centrosomes involved in microtubule orgainzation. *Cell* **76**, 639–650 (1994).
37. Nigg, E. A. Cell division mitotic kinases as regulators of cell division and its checkpoints. *Nature Rev. Mol. Cell Biol.* **2**, 21–32 (2001).
38. Donaldson, M. M., Tavares, A. A., Ohkura, H., Deak, P. & Glover, D. M. Metaphase arrest with centromere separation in *polo* mutants of *Drosophila*. *J. Cell Biol.* **153**, 663–676 (2001).
39. Do Carmo Avides, M., Tavares, A. & Glover, D. M. Polo kinase and Asp are needed to promote the mitotic organizing activity of centrosomes. *Nature Cell Biol.* **3**, 421–424 (2001).
Shows that Polo kinase and Asp are in the same complex, that Polo phosphorylates Asp *in vitro* and that both active Polo and phosphorylation of Asp are required for recruitment of γ-tubulin complexes to centrosomes.
40. Feng, Y. *et al.* Association of polo-like kinase with α-, β- and γ-tubulins in a stable complex. *Biochem. J.* **339**, 435–442 (1999).
41. Mogensen, M. M., Mackie, J. B., Doxsey, S. J., Stearns, T. & Tucker, J. B. Centrosomal deployment of γ-tubulin and pericentrin: evidence for a microtubule-nucleating domain and a minus-end docking domain in certain mouse epithelial cells. *Cell Motil. Cytoskel.* **36**, 276–290 (1997).
Demonstration that unique microtubule minus-end anchoring sites exist in epithelial cells that lack proteins involved in microtubule nucleation.
42. Mogensen, M. M., Malik, A., Piel, M., Bouckson-Castaing, V. & Bornens, M. Microtubule minus-end anchorage at centrosomal and non-centrosomal sites: the role of ninein. *J. Cell Sci.* **113**, 3013–3023 (2000).
Identification of a protein ninein that localizes to unique microtubule minus-end anchoring sites in epithelial cells and to specializations on the maternal centriole involved in microtubule anchoring (also see reference 41).
43. Gromley, A. *et al.* A novel centriolar protein with a centrosomal function. *Mol. Biol. Cell* **11**, 90 (2000).
44. Mogensen, M. M. Microtubule release and capture in epithelial cells. *Biol. Cell* **91**, 331–341 (1999).
45. Chretien, D., Buendia, B., Fuller, S. D. & Karsenti, E. Reconstruction of the centrosome cycle from electron micrographs. *J. Struct. Biol.* **120**, 117–133 (1997).
46. Mitchison, T. & Salmon, E. D. Poleward kinetochore movement occurs during both metaphase and anaphase-A in newt lung cells mitosis. *J. Cell Biol.* **119**, 569–582 (1992).
47. Keating, T. J., Peloquin, J. G., Rodionove, V., Momcilovic, D. & Borisy, G. G. Microtubule release from the centrosome. *Proc. Natl Acad. Sci. USA* **94**, 5078–5083 (1997).
Visualization of microtubule release from centrosomes by time-lapse microscopy.
48. Hartman, J. *et al.* Katanin, a microtubule-severing protein, is a novel AA ATPase that targets to the centrosome using a WD40-containing subunit. *Cell* **93**, 277–287 (1998).
49. Ahmad, F. J., Yu, W., McNally, F. J. & Baas, P. W. An essential role for katanin in severing microtubules in the neuron. *J. Cell Biol.* **145**, 305–315 (1999).
50. Desai, A., Verma, S., Mitchison, T. J. & Walczak, C. E. Kin I kinesins are microtubule-destabilizing enzymes. *Cell* **96**, 69–78 (1999).
51. Purohit, A., Tynan, S. H., Vallee, R. & Doxsey, S. J. Direct interaction of pericentrin with cytoplasmic dynein light intermediate chain contributes to mitotic spindle organization. *J. Cell Biol.* **147**, 481–491 (1999).
Demonstration that pericentrin interacts directly with a component of cytoplasmic dynein, the light intermediate chain (LIC), suggesting a role for LIC in centrosome assembly.
52. Tynan, S. H., Purohit, A., Doxsey, S. J. & Vallee, R. B. Light intermediate chain 1 defines a functional subfraction of cytoplasmic dynein which binds to pericentrin. *J. Biol. Chem.* **275**, 32763–32768 (2000).
53. Merdes, A., Ramyar, K., Vechio, J. D. & Cleveland, D. W. A complex of NuMA and cytoplasmic dynein is essential for mitotic spindle assembly. *Cell* **87**, 447–458 (1996).
54. Balczon, R., Varden, C. E. & Schroer, T. A. Role for microtubules in centrosome doubling in Chinese hamster ovary cells. *Cell Motil. Cytoskel.* **42**, 60–72 (1999).
55. Young, A., Dictenberg, J., Purohit, A., Tuft, R. & Doxsey, S. Dynein-mediated assembly of pericentrin and γ-tubulin onto centrosomes. *Mol. Biol. Cell* **11**, 2047–2056 (2000).
56. Merdes, A., Heald, R., Samejima, K., Earnshaw, W. C. & Cleveland, D. W. Formation of spindle poles by dynein/dynactin-dependent transport of NuMA. *J. Cell Biol.* **149**, 851–862 (2000).
57. Kubo, A., Sasaki, H., Yuba-Kubo, A., Tsukita, S. & Shiina, N. Centriolar satellites: molecular characterization, ATP-dependent movement toward centrioles and possible involvement in ciliogenesis. *J. Cell Biol.* **147**, 969–980 (1999).
Shows cytoplasmic dynein-dependent recruitment of pericentrin and PCM-1, to centrosomes, as well as time-lapse imaging of these movements.
58. Felix, M.-A., Antony, C., Wright, M. & Maro, B. Centrosome assembly *in vitro*: role of γ-tubulin recruitment in *Xenopus* sperm aster formation. *J. Cell Biol.* **124**, 19–31 (1994).
59. Schnackenberg, B. J., Khodjakov, A., Rieder, C. L. & Palazzo, R. E. The disassembly and reassembly of functional centrosomes *in vitro*. *Proc. Natl Acad. Sci. USA* **95**, 9295–9300 (1998).
60. Khodjakov, A. & Rieder, C. L. The sudden recruitment of γ-tubulin to the centrosome at the onset of mitosis and its dynamic exchange throughout the cell cycle, do not require microtubules. *J. Cell Biol.* **146**, 585–596 (1999).
Shows microtubule-independent recruitment of γ-tubulin to centrosomes.
61. Piel, M., Nordberg, J., Euteneuer, U. & Bornens, M. Centrosome-dependent exit of cytokinesis in animal cells. *Science* **291**, 1550–1553 (2001).
Suggests a novel requirement for centrioles in the completion of cytokinesis.
62. Piel, M., Meyer, P., Khodjakov, A., Rieder, C. L. & Bornens, M. The respective contributions of the mother and daughter centrioles to centrosome activity and behavior in vertebrate cells. *J. Cell Biol.* **149**, 317–330 (2000).
Shows that maternal and daughter centrioles are dynamic structures that nucleate microtubules, but only the maternal centriole retains, and presumably anchors them.
63. Hyman, A. & Karsenti, E. The role of nucleation in patterning microtubule networks. *J. Cell Sci.* **111**, 2077–2083 (1998).
64. Compton, D. A. Spindle assembly in animal cells. *Annu. Rev. Biochem.* **69**, 95–114 (2000).
65. Megraw, T. L., Kao, L. R. & Kaufman, T. C. Zygotic development without functional mitotic centrosomes. *Curr. Biol.* **11**, 116–120 (2001).
Suggests that centrosomes and astral microtubules are not required for *Drosophila* development.
66. Hinchcliffe, E. H., Miller, F. J., Cham, M., Khodjakov, A. & Sluder, G. Requirement of a centrosomal activity for cell cycle progression through G1 into S phase. *Science* **291**, 1547–1550 (2001).
Shows that surgical removal of centrosomes does not affect spindle assembly but inhibits progression

from G1 to S phase.
67. Khodjakov, A., Cole, R. W., Oakley, B. R. & Rieder, C. L. Centrosome-independent mitotic spindle formation in vertebrates. *Curr. Biol.* **10**, 59–67 (2000).
Shows that laser ablation of centrosomes in vertebrate cells does not prevent spindle assembly.
68. Heald, R., Regis, T., Habermann, A., Karsenti, E. & Hyman, A. Spindle assembly in *Xenopus* egg extracts: respective roles of centrosomes and microtubule self-organization. *J. Cell Biol.* **138**, 615–628 (1997).
Shows that centrosomes act dominantly in spindle assembly.
69. Segal, M. & Bloom, K. Control of spindle polarity and orientation in *Saccharomyces cerevisiae*. *Trends Cell Biol.* **11**, 160–166 (2001).
70. Doe, C. Q. & Bowerman, B. Asymmetric cell division: fly neuroblast meets worm zygote. *Curr. Opin. Cell Biol.* **13**, 68–75 (2001).
71. Schuyler, S. C. & Pellman, D. Search, capture and signal: games microtubules and centrosomes play. *J. Cell Sci.* **114**, 247–255 (2001).
72. Schuyler, S. C. & Pellman, D. Microtubule 'plus-end-tracking proteins': the end is just the beginning. *Cell* **105**, 421–424 (2001).
73. O'Connell, C. B. & Wang, Y. L. Mammalian spindle orientation and position respond to changes in cell shape in a dynein-dependent fashion. *Mol. Biol. Cell* **11**, 1765–1774 (2000).
74. Grill, S. W., Gönczy, P., Stelzer, E. H. & Hyman, A. A. Polarity controls forces governing asymmetric spindle positioning in the *Caenorhabditis elegans* embryo. *Nature* **409**, 630–633 (2001).
75. Khodjakov, A. & Rieder, C. L. Centrosomes enhance the fidelity of cytokinesis in vertebrates and are required for cell cycle progression. *J. Cell Biol.* **153**, 237–242 (2001).
Shows that laser ablation of centrosomes causes defects in cytokinesis and blocks cell cycle progression from G1 to S phase.
76. Cottingham, F. R. & Hoyt, M. A. Mitotic spindle positioning in *Saccharomyces cerevisiae* is accomplished by antagonistically acting microtubule motor proteins. *J. Cell Biol.* **138**, 1041–1053 (1997).
77. Gachet, Y., Tournier, S., Millar, J. B. & Hyams, J. S. A MAP kinase-dependent actin checkpoint ensures proper spindle orientation in fission yeast. *Nature* **412**, 352–355 (2001).
78. Yin, H., Pruyne, D., Huffaker, T. C. & Bretscher, A. Myosin V orientates the mitotic spindle in yeast. *Nature* **406**, 1013–1015 (2000).
79. Giansanti, M. G., Gatti, M. & Bonaccorsi, S. The role of centrosomes and astral microtubules during asymmetric division of *Drosophila* neuroblasts. *Development* **128**, 1137–1145 (2001).
80. Roeglers, F., Younger-Shepherd, S., Jan, L. Y. & Jan, Y. N. Two types of asymmetric divisions in the *Drosophila* sensory organ precursor cell lineage. *Nature Cell Biol.* **3**, 58–67 (2001).
81. Rappaport, R. Establishment of the mechanism of cytokinesis in animal cells. *Int. Rev. Cytol.* **105**, 245–281 (1986).
82. Adames, N. R. & Cooper, J. A. Microtubule interactions with the cell cortex causing nuclear movements in *Saccharomyces cerevisiae*. *J. Cell Biol.* **149**, 863–874 (2000).
83. Bardin, A. J., Visintin, R. & Amon, A. A mechanism for coupling exit from mitosis to partitioning of the nucleus. *Cell* **102**, 21–31 (2000).
84. Bloecher, A., Venturi, G. M. & Tatchell, K. Anaphase spindle position is monitored by the BUB2 checkpoint. *Nature Cell Biol.* **2**, 556–558 (2000).
85. Gruneberg, U., Campbell, K., Simpson, C., Grindlay, J. & Schiebel, E. Nud1p links astral microtubule organization and the control of exit from mitosis. *EMBO J.* **19**, 6475–6488 (2000).
86. Pereira, G., Hofken, T., Grindlay, J., Manson, C. & Schiebel, E. The Bub2p spindle checkpoint links nuclear migration with mitotic exit. *Mol. Cell* **6**, 1–10 (2000).
References 83 to 86 describe the identification and characterization of a novel checkpoint that monitors movement of the yeast spindle pole body into the bud at the end of mitosis.
87. Doxsey, S. J. Centrosomes as command centres for cellular control. *Nature Cell Biol.* **3**, E105–E108 (2001).
88. Pereira, G. & Schiebel, E. The role of the yeast spindle pole body and the mammalian centrosome in regulating late mitotic events. *Curr. Opin. Cell Biol.* (in the press).
89. Andreassen, P. R., Lohez, O. D., Lacroix, F. B. & Margolis, R. L. Tetraploid state induces p53-dependent arrest of nontransformed mammalian cells in G1. *Mol. Biol. Cell* **12**, 1315–1328 (2001).
90. Liu, J., Wang, H. & Balasubramanian, M. K. A checkpoint that monitors cytokinesis in *Schizosaccharomyces pombe*. *J. Cell Sci.* **113**, 1223–1230 (2000).
91. McCollum, D. & Gould, K. L. Timing is everything: regulation of mitotic exit and cytokinesis by the MEN and SIN. *Trends Cell Biol.* **11**, 89–95 (2001).
92. Hinchcliffe, E. H., Li, C., Thompson, E. A., Maller, J. L. & Sluder, G. Requirement of Cdk2–cyclin E activity for repeated centrosome reproduction in *Xenopus* egg extracts. *Science* **283**, 851–854 (1999).
93. Lacey, K. R., Jackson, P. K. & Stearns, T. Cyclin-dependent kinase control of centrosome duplication. *Proc. Natl Acad. Sci. USA* **96**, 2817–2822 (1999).
References 92 and 93 report the identification of Cdk2–cyclinE/A as regulators of centrosome duplication in *Xenopus* embryos and extracts.
94. Stevenson, V. A., Kramer, J., Kuhn, J. & Theurkauf, W. E. Centrosomes and the Scrambled protein coordinate microtubule-independent actin reorganization. *Nature Cell Biol.* **3**, 68–75 (2001).
Microtubule-independent centrosome control of actin deposition in *Drosophila* embryos.
95. Scott, M. P. & O'Farrell, P. H. Spatial programming of gene expression in early *Drosophila* embryogenesis. *Annu. Rev. Cell Biol.* **2**, 49–80 (1986).
96. Sibon, O. C., Kelkar, A., Lemstra, W. & Theurkauf, W. E. DNA-replication/DNA-damage-dependent centrosome inactivation in *Drosophila* embryos. *Nature Cell Biol.* **2**, 90–95 (2000).
Loss of microtubule nucleation and γ-tubulin from centrosomes after DNA damage.
97. Hinchcliffe, E. H. & Sluder, G. 'It takes two to tango': understanding how centrosome duplication is regulated throughout the cell cycle. *Genes Dev.* **15**, 1167–1181 (2001).
98. Lange, B. M. & Gull, K. A molecular marker for centriole maturation in the mammalian cell cycle. *J. Cell Biol.* **130**, 919–927 (1995).
99. Chang, P. & Stearns, T. δ-tubulin and ε-tubulin: two new human centrosomal tubulins reveal new aspects of centrosome structure and function. *Nature Cell Biol.* **2**, 30–35 (2000).
Identification of two novel centrosome-associated tubulins in humans, δ and ε.
100. Paoletti, A., Moudjou, M., Paintrand, M., Salisbury, J. L. & Bornens, M. Most of centrin in animal cells is not centrosome-associated and centrosomal centrin is confined to the distal lumen of centrioles. *J. Cell Sci.* **109**, 3089–3102 (1996).
101. Ruiz, F., Beisson, J., Rossier, J. & Dupuis-Williams, P. Basal body duplication in *Paramecium* requires γ-tubulin. *Curr. Biol.* **9**, 43–46 (1999).
102. Palazzo, R. E., Vaisberg, E., Cole, R. W. & Rieder, C. L. Centriole duplication in lysates of *Spisula solidissima* oocytes. *Science* **256**, 219–221 (1992).
103. Marshall, W. F., Vucica, Y. & Rosenbaum, J. L. Kinetics and regulation of *de novo* centriole assembly. Implications for the mechanism of centriole duplication. *Curr. Biol.* **11**, 308–317 (2001).
Demonstration of centriole/basal body formation *de novo* in *Spisula* and *Chlamydomonas*.
104. Andersen, S. S. Molecular characteristics of the centrosome. *Int. Rev. Cytol.* **187**, 51–109 (1999).
105. Fry, A. M., Meraldi, P. & Nigg, E. A. A centrosomal function for the human Nek2 protein kinase, a member of the NIMA family of cell cycle regulators. *EMBO J.* **17**, 470–481 (1998).
Identification of a kinase that seems to be involved in centrosome splitting in G2/M.
106. Mayor, T., Stierhof, Y. D., Tanaka, K., Fry, A. M. & Nigg, E. A. The centrosomal protein C-Nap1 is required for cell cycle-regulated centrosome cohesion. *J. Cell Biol.* **151**, 837–846 (2000).
107. Sharp, D. J., Rogers, G. C. & Scholey, J. M. Microtubule motors in mitosis. *Nature* **407**, 41–47 (2000).
108. Matsumoto, Y., Hayashi, K. & Nishida, E. Cyclin-dependent kinase 2 (Cdk2) is required for centrosome duplication in mammalian cells. *Curr. Biol.* **9**, 429–432 (1999).
109. Meraldi, P., Lukas, J., Fry, A. M., Bartek, J. & Nigg, E. A. Centrosome duplication in mammalian somatic cells requires E2F and Cdk2–cyclin A. *Nature Cell Biol.* **1**, 88–93 (1999).
References 108 and 109 describe the identification of Cdk2–cyclinE/A as regulators of centrosome duplication in mammalian cells.
110. Fisk, H. A. & Winey, M. The mouse Mps1p-like kinase regulates centrosome duplication. *Cell* **106**, 95–104 (2001).
111. Okuda, M. *et al.* Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. *Cell* **103**, 127–140 (2000).
112. Tokuyama, Y., Horn, H. F., Kawamura, K., Tarapore, P. & Fukasawa, K. Specific phosphorylation of nucleophosmin on Thr199 by cyclin-dependent kinase 2-cyclin E and its role in centrosome duplication. *J. Biol. Chem.* **276**, 21529–21537 (2001).
113. Tarapore, P., Horn, H. F., Tokuyama, Y. & Fukasawa, K. Direct regulation of the centrosome duplication cycle by the p53-p21(Waf1/Cip1) pathway. *Oncogene* **20**, 3173–3184 (2001).
114. Freed, E. *et al.* Components of an SFC ubiquitin ligase localize to centrosomes and regulate the centrosome duplication cycle. *Genes Dev.* **13**, 2242–2257 (1999).
Demonstrates a role for the ubiquitin-mediated degradation pathway in centrosome duplication.
115. Nakayama, K. *et al.* Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. *EMBO J.* **19**, 2069–2081 (2000).
116. Wojcik, E. J., Glover, D. M. & Hays, T. S. The SCF ubiquitin ligase protein slimb regulates centrosome duplication in *Drosophila*. *Curr. Biol.* **10**, 1131–1134 (2000).
117. O'Connell, K. F. *et al.* The *C. elegans zyg-1* gene encodes a regulator of centrosome duplication with distinct maternal and paternal roles in the embryo. *Cell* **105**, 547–558 (2001).
118. Wakefield, J. G., Huang, J. Y. & Raff, J. W. Centrosomes have a role in regulating the destruction of cyclin B in early *Drosophila* embryos. *Curr. Biol.* **10**, 1367–1370 (2000).
119. Clute, P. & Pines, J. Temporal and spatial control of cyclin B1 destruction in metaphase. *Nature Cell Biol.* **1**, 82–87 (1999).
References 118 and 119 demonstrate that cyclin degradation is spatially regulated, beginning on centrosomes/spindle poles and moving up the spindle to the chromosomes.
120. Diviani, D. & Scott, J. D. AKAP signaling complexes at the cytoskeleton. *J. Cell Sci.* **114**, 1431–1437 (2001).
121. Feliciello, A., Gottesman, M. E. & Avvedimento, E. V. The biological functions of A-kinase anchor proteins. *J. Mol. Biol.* **308**, 99–114 (2001).
122. Groisman, I. *et al.* CPEB, maskin, and cyclin B1 mRNA at the mitotic apparatus: implications for local translational control of cell division. *Cell* **103**, 435–447 (2000).
123. Brinkley, B. R. & Goepfert, T. M. Supernumerary centrosomes and cancer: Boveri's hypothesis resurrected. *Cell Motil. Cytoskel.* **41**, 281–288 (1998).
124. Marx, J. Cell biology. Do centrosome abnormalities lead to cancer? *Science* **292**, 426–429 (2001).
125. Pihan, G. A. *et al.* Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression. *Cancer Res.* **61**, 2212–2219 (2001).
126. Rosenbaum, J. L., Cole, D. G. & Diener, D. R. Intraflagellar transport: the eyes have it. *J. Cell Biol.* **144**, 385–388 (1999).
127. Quintyne, N. J. *et al.* Dynactin is required for microtubule anchoring at centrosomes. *J. Cell Biol.* **147**, 1–14 (1999).
128. Pihan, G. A. *et al.* Centrosome defects and genetic instability in malignant tumors. *Cancer Res.* **58**, 3974–3985 (1998).

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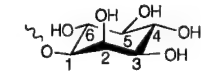
ERRATUM

HOW PROTEINS MOVE LIPIDS AND LIPIDS MOVE PROTEINS

Sprong, H., van der Sluijs, P. and van Meer G.

Nature Rev. Mol. Cell Biol. **2**, 504–513 (2001).



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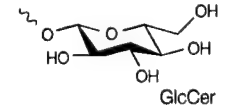

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Centrosomes as command centres for cellular control

Stephen J. Doxsey

For almost a century, our understanding of the tiny structure at the centre of the cell called the centrosome was limited to its ability to organize mitotic spindles and other microtubule arrays. However, recent studies have indicated new roles for centrosomes in cytokinesis and cell-cycle progression.

The centrosome is positioned in the centre of interphase cells at the focus of a radial array of microtubules. Microtubules are polymers composed of α- and β-tubulin subunits, and their growth is initiated in the peripheral region of the centrosome (the pericentriolar matrix) by a complex containing a related protein called γ-tubulin[1,2]. At the centrosome core lies a pair of specialized barrel-shaped microtubule assemblies of unknown function called centrioles. The centrioles and surrounding pericentriolar matrix define the centrosome as one of the most complex non-membranous organelles in the cell. Despite their structural and molecular complexity, the only well-characterized function of centrosomes is to nucleate and organize polarized microtubule arrays that generate cell polarity and form the structural framework of the meiotic and mitotic spindles. An emerging frontier in centrosome biology concerns the role of this organelle in anchoring regulatory molecules through interactions with scaffold proteins[3,4]. Despite a rapidly growing list of such centrosome-associated regulatory proteins and activities, there have been very few direct links between their localization to the centrosome and specific cellular functions. However, three recent papers[5–7] provide such a link. Using different experimental approaches, the three studies have provided evidence to link centrosome activity to the completion of cell division (cytokinesis) and the activation of DNA replication (entry into S phase).

The central role of the mitotic spindle in defining the site of cell cleavage during cytokinesis has long been known[8]. Hinchcliffe *et al.*[5] used microsurgery to remove the centrosome and then assayed the mitotic consequences by time-lapse video microscopy. Previous analysis of acentrosomal cells using fixed time points and static images indicated that they arrest in mitosis[9]. However, continuous monitoring by video microscopy demonstrated that acentrosomal cells entered mitosis and formed mitotic spindles. After this, most cells were delayed in mitosis before chromosome segregation, and many failed to

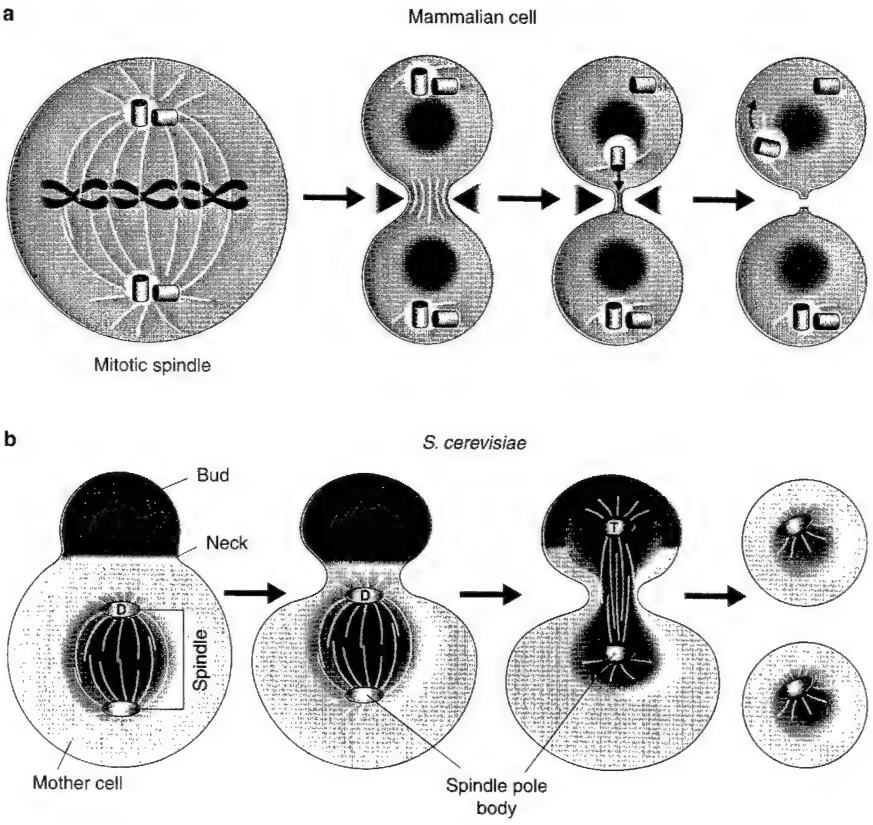

Figure 1 **Positioning of centrosomes and spindle pole bodies during exit from cytokinesis. a, A mitotic spindle in mammalian cells (left) with two centrosomes, each with two centrioles (rectangles). The pericentriolar matrix around the maternal centriole (yellow) nucleates microtubules (white). As cytokinesis progresses (right), nuclei reform in the daughter cells (blue), the cell constricts and microtubules remain in an intercellular bridge between cells. At telophase (second left) the cytokinesis checkpoint (red arrows) is turned on. The maternal centriole containing microtubule-nucleating activity moves to the bridge (arrow). The bridge constricts and microtubules depolymerize. The checkpoint is still on. The maternal centriole moves away and back to the centrosome (arrow). The checkpoint is turned off and the cell cleaves. b, A mitotic spindle in the mother cell of a budding yeast (*Saccharomyces cerevisiae*) has an inactive (GDP-bound) form of the GTP-binding protein Tem1 on a spindle pole body facing the bud neck. The mitotic exit (cytokinesis) checkpoint is on (left). The spindle nears the bud neck. Cytoplasmic microtubules contact the neck and may maintain an active checkpoint (which is still on). The spindle moves into the bud containing active protein (green). Microtubules are destabilized or lose contact with the bud. SPB-associated Tem1 is activated by a GTP exchange factor (Lte1) in the bud (green) from the inactive GDP form (D) to the active GTP form (T). Microtubules destabilize and no longer interact with the bud neck. The checkpoint is off and mitotic exit is initiated, resulting in cell cleavage and the generation of two daughter cells (right).**

complete cytokinesis. These cells remained attached by thin intercellular bridges or aborted cytokinesis to form single binucleate cells. Hinchcliffe and colleagues conclude that the mitotic delays are attributable to inefficient organization of mitotic spindles in cells lacking 'core' centrosome components such as centrioles and associated proteins. Cytokinesis defects were also observed in acentrosomal cells by Khodakov and Reider[6], who used high-energy lasers to destroy centrosomes tagged with a chimaeric protein composed of γ-tubulin and green fluorescent protein (GFP). When both centrosomes in mitotic spindles were destroyed, astral microtubules were lost, spindles became misorientated and chromosomes were unable to move out of the path of the cleaving cell. Thus, failure of cytokinesis might result from physical obstruction of the cleavage furrow.

An alternative and intriguing explanation for cleavage failure in both of these acentrosomal systems is proposed by Piel *et al.*[7]. Using time-lapse microscopy, they examined the behaviour of centrosomes labelled with centrin coupled to GFP. They found that the final cell-cleavage event is tightly correlated with centriole movement and specifically with movement of the maternal centriole, the older of the two centrioles. It serves as the template for the formation of the younger daughter centriole and, in this study, incorporated more GFP–centrin and was labelled more brightly than the daughter. The maternal centriole always moved to the intercellular bridge before the bridge narrowed and the microtubules within were depolymerized. When the centriole moved away, the cell proceeded to cleave. Furthermore, *Drosophila* cells lacking centrioles exhibited an extremely high rate of cytokinesis failure, and changes in the adherent properties or confluency of cells that diminished the efficiency of centriole movement to the bridge also delayed cell cleavage. These findings indicate that movement of the maternal centriole to the intercellular bridge may be required for completion of cytokinesis.

Is it possible that a common 'thread' connects all three sets of findings? Careful examination of images presented in the three papers reveals thin thread-like bridges between postmitotic acentrosomal cells. These could represent persistent connections between cells that have not completed cytokinesis. The remaining cells might have divided by an alternative mechanism, termed 'traction-mediated cytokinesis'[10], whereby cells crawl apart and break in two. This type of cleavage is unlikely to occur *in vivo*, where cells within tissues are not free to move greatly relative to one another. Thus, true failure of cytokinesis could be more extensive than the data would indicate. The lack of centrioles could therefore prevent cytokinesis in all acentrosomal cell systems examined.

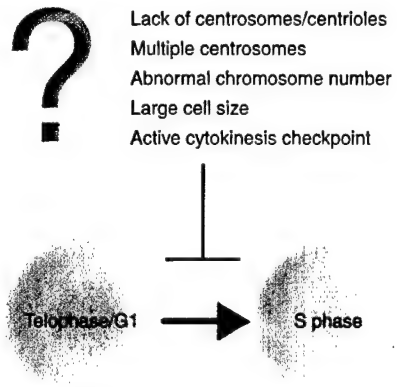

Figure 2 **Potential activators of G1 arrest in mammalian cells with an altered centrosome number.**

These results indicate two mechanisms by which centrosomes might regulate the final stages of cytokinesis. Centrosomes could directly activate cytokinesis or could release cells from arrest at a cytokinesis checkpoint. Recent studies with both budding and fission yeast[11–14] favour the latter possibility (Fig. 1). During the final stages of cytokinesis in budding yeast, the mitotic spindle moves from the mother cell into the bud neck (the bridge between the mother and nascent daughter cell). During this process, the leading spindle pole body (equivalent to the mitotic centrosome) enters the bud, carrying an inactive form of Tem1, a GTP-binding protein that regulates exit from mitosis. The Tem1-exchange factor Lte1 is confined to the bud and is proposed to activate Tem1 and trigger a signalling pathway that mediates completion of cytokinesis and exit from mitosis. A more recent study has shown that further events might be required for release from the cytokinesis checkpoint, most notably microtubule depolymerization at the bud neck (the equivalent of the intercellular bridge) and loss of contact between microtubules and the bud neck[15], similar to that seen in mammalian cells[7]. These observations indicate that there might be many common elements in the pathways of yeast and mammals that mediate the completion of cytokinesis. Identification and functional analysis of mammalian homologues of the yeast components involved in mitotic exit should help to identify the function of the cytokinesis checkpoint in mammalian cells.

Hinchcliffe and colleagues took the acentrosomal cells produced by microsurgery[5] and monitored them after mitosis (or failure in cytokinesis); most seemed to arrest in G1 phase and did not initiate DNA replication. In contrast, control cells that were cut to remove an equivalent volume of cytoplasm without removing the centrosome replicated their DNA normally and continued to divide. Similar results were obtained in laser-ablation studies by Khodjakov and Reider, who eliminated one centrosome of the mitotic pair, giving rise to acentrosomal and centrosome-containing daughter cells after cytokinesis. The centrosome-containing progeny progressed into S phase, whereas acentrosomal cells did not and seemed to arrest in G1 phase.

One interpretation of the fact that DNA replication was blocked is that centrosomes directly activate or concentrate factors that are essential for the initiation of DNA synthesis. Consistent with this idea is the centrosomal localization of molecules that control entry into S phase[16,17]. An alternative explanation is that animal cells monitor the presence of centrosomes, and in their absence activate a checkpoint that prevents the onset of S phase. The pathway that activates G1 arrest in acentrosomal cells has not yet been characterized. It is likely to be complicated by potential downstream effects of centrosome loss such as mitotic defects, chromosome missegregation, cytokinesis failure and activation of a cytokinesis checkpoint. Each of these problems has the potential to induce G1 arrest on its own. For example, in fission yeast, failure of cytokinesis triggers a checkpoint that inhibits progression of the nuclear cell cycle, albeit at a later stage in the cell cycle (G2 phase) (ref. 14, and see note added in proof). Similarly, the absence of centrosomes in animal cells could lead to the failure of cytokinesis as a result of defects in positioning of the mother centriole[7], and these cleavage failures could then trigger a G1 arrest through a cytokinesis checkpoint. This idea is supported by the findings of Andreasson *et al.*[18], who have shown that cells that are blocked in cytokinesis, without disrupting spindle function or chromosome segregation, subsequently arrest in G1 phase as tetraploid cells. This rules out the possibility that the G1 arrest occurs in response to spindle defects, but does not exclude potential cellular responses to excess centrosomes, excess chromosomes, increased cell size or activation of a cytokinesis checkpoint (Fig. 2). Many of the potential contributors to G1 arrest are present in both tetraploid and acentrosomal cells. If G1 arrest in acentrosomal cells results from a lack of centrosomes or downstream events, S-phase progression could be restored by the microinjection of centrosomes. Regardless of the mechanism, coupling of the centrosome cycle to the cell cycle could ensure that the appropriate number of functional centrosomes is maintained in animal cells. This might have important implications in cancer, in which the presence of excessive numbers of centrosomes at early tumour stages[19] could contribute to spindle defects, genetic instability and tumour progression, either alone

or in combination with other cellular defects and mutations[20].

The work described here indicates that the centrosome in mammalian cells might have an essential function in cytokinesis and progression from G1 into S phase. It is unclear whether centrosomes mediate these events directly, or whether centrosome defects trigger checkpoints that monitor cell-cycle progression. A better understanding of the mechanisms by which centrosomes influence these events will come from an appreciation of the molecular details of the pathways involved. One approach to this problem will be to identify and analyse proteins that are homologous to those involved in similar pathways in yeast. Another is to investigate further the centrosome components that have already been identified in metazoans. It has been proposed that some of the integral centriole/centrosome proteins that have been identified as autoantibody targets in autoimmune diseases might function in cell-cycle progression (ref. 21; S. Doxsey and R. Balczon, unpublished observations). Further regulatory pathways might also be influenced by centrosomes, and the centrosomal localization of regulatory molecules that communicate with signal-transduction pathways indicates a possible connection between centrosomes and the extracellular environment. Elucidation of the links between centrosomes and these and other cellular pathways is likely to be a fertile area of future discovery.

Note added in proof: Additional references are refs 22 and 23. □

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1. Zheng, Y., Wong, M. L., Alberts, B. & Mitchison, T. *Nature* **378**, 578–583 (1995).
2. Moritz, M., Braunfield, M. B., Sedat, J. W., Alberts, B. & Agard, D. A. *Nature* **378**, 638–640 (1995).
3. Zimmerman, W., Sparks, C. A., & Doxsey, S. J. *Curr. Opin. Cell Biol.* **11**, 122–128 (1999).
4. Diviani, D. & Scott, J. D. *J. Cell Sci.* **114**, 1431–1437 (2001).

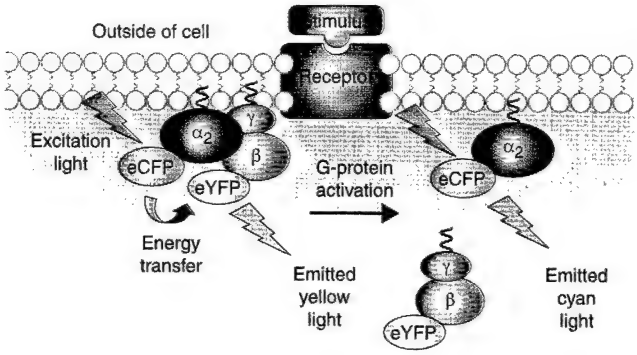
G-protein activation live

G proteins are commonly used in signal transduction pathways in animal cells in response to diverse stimuli such as light, odorants, hormones, neurotransmitters and chemoattractants. They consist of heterotrimers of an α-subunit, a β-subunit and a γ-subunit. Upon ligand binding, G-protein-coupled receptors (GPCRs) catalyse the exchange of GDP to GTP on the α-subunit. The GTP-bound α-subunit is then thought to dissociate from the βγ-heterodimer, allowing each party to signal to its downstream effector. The activation is terminated by GTP hydrolysis by the GTPase domain of the α-subunit, leaving the α-subunit in the GDP-bound form and allowing heterotrimerization to resume.

Activation of heterotrimeric G proteins has so far been difficult to monitor directly *in vivo* in real time because of technical difficulties. Janetopoulos and colleagues now use fluorescence resonance energy transfer (FRET) technology to study the cycle of activation of heterotrimeric G proteins in time and space *in vivo*, shedding light on the kinetics and subcellular localization of heterotrimeric G proteins (*Science* **291**, 2408–2411; 2001).

G proteins in the social amoeba *Dictyostelium discoideum* are activated by the binding of the chemoattractant cAMP to its receptor cAR1. This results in the recruitment and activation of effectors of polarized actin polymerization, membrane protrusion at the leading edge of the cell and cell migration towards the chemoattractant. Janetopoulos and colleagues expressed fusion constructs of the $G\alpha_2$ subunit and cyan fluorescent protein (CFP) and of the Gβ subunit and yellow fluorescent protein (YFP) in a $G\alpha_2$- or Gβ-null background. These fusion proteins were shown to be fully functional in terms of signalling. On exposure to a light with a wavelength of 440 nm, which excites the CFP fluorophore, energy is transferred from CFP to YFP when the two probes are in close proximity, and a fluorescent signal is emitted by the yellow fluorophore. When the fluorophores are too far apart, the energy cannot be transferred from CFP to YFP and there is no fluorescence emission from the YFP probe.

The interaction between the α- and β-subunits was thus monitored by exposing cells to a wavelength of 440 nm and recording a FRET fluorescence signal between 460 and 600 nm. A FRET fluorescence emission signal was detected at 527 nm specifically in cells expressing both fusion constructs. Activation by cAMP results in a sharp decrease in FRET fluorescence, which suggests complete dissociation of the heterotrimer rather than just a change in conformation. The kinetics of activation is extremely rapid: maximum stimulation was reached less than 10 seconds after the addition of cAMP, and 90% of maximum activation was achieved in a matter of a few seconds. Activation is reversible, as the removal of cAMP resulted in a return to maximum fluorescence levels within 2 minutes. It is also dose-dependent: the EC_{50} value for cAMP was estimated to be 10 nM. The k_d for binding of cAMP to cAMP receptor-1 was approximately 180 nM, suggesting that the steady-state level of G-protein activation saturates before all receptors are occupied.



COURTESY OF C. JANETOPOULOS AND P. DEVREOTES

Sustained activation of GCPRs is known to result in adaptation, for example, of actin polymerization and other responses. As Janetopoulos and colleagues found that sustained exposure to cAMP leads to a prolonged loss of the FRET fluorescence signal, the mechanism of adaptation does not seem to be at the level of G proteins; it must be farther downstream.

Taken together, these observations also suggest that occupied receptors repeatedly activate G proteins, that regulators of G-protein signalling (RGS) are likely to modulate the ratio of active to inactive G proteins rather than the time course of activation, and that the distribution of activated G proteins probably reflects the shallow gradient of receptor occupancy rather than the sharply localized physiological response at the leading edge of the cell.

VALERIE DEPRAETERE

5. Hinchcliffe, E. H., Miller, F. J., Cham, M., Khodjakov, A. & Sluder, G. *Science* **291**, 1547–1550, (2001).
6. Khodjakov & Reider, {AU: authors' initials?} *J. Cell Biol.* **153**, 237–242 (2001).
7. Piel, M., Nordberg, J., Euteneuer, U. & Bornens, M. *Science* **291**, 1550–1553 (2001).
8. Rappaport, R. *Int. Rev. Cytol.* **105**, 2123–2135 (1986).
9. Maniotis, A. & Schliwa, M. *Cell* **67**, 495–504 (1991).
10. Burton, K. & Taylor, D. L. *Nature* **385**, 450–453 (1997).
11. Bardin, A. J, Visitin, R. & Amon, A. *Cell* **102**, 21–31 (2000).
12. Bloecher, A., Venturi, G. M. & Tatchell, K. *Nature Cell Biol.* **2**, 556–558 (2000).
13. Perierra, G., Hofken, T., Grindlay, J., Manson, C. & Schiebel, E. *Mol. Cell* **6**, 1–10 (2000).
14. McCollum, D. & Gould, K. L. *Trends Cell Biol.* **11**, 89–95 (2001).
15. Adames, N. R., Oberle, J. R. & Cooper, J. A. *J. Cell Biol.* **153**, 159–168 (2001).
16. Hinchcliffe, E. H., Thompson, E. A., Maller, J. L. & Sluder, G. *Science* **283**, 851–854 (1999).
17. Lacey, K. R., Jackson, P. K. & Stearns, T. *Proc. Natl Acad. Sci. USA* **96**, 2817–2822 (2000).
18. Andreassen, P. R., Lohez, O. D., Lacroix, F. B. & Margolis, R. L. *Mol. Biol. Cell* (in the press).
19. Pihan, G. *et al. Cancer Res.* **61**, 2212–2219 (2001).
20. Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S. & van de Woude, G. F. *Science* **271**, 1744–1747 (1996).
21. Purohit, A., Tynan, S. H., Vallee, R. & Doxsey, S. J. *J. Cell Biol.* **147**, 481–491 (1999).
22. Le Goff, X., Woollard, A. & Simanis, V. *Mol. Gen. Genet.* **262**, 163–172 (1999).
23. Liu, J., Wang, H. & Balasubramanian, M. K. *J. Cell Sci.* **113**, 1223–1230 (2000).

Caveolae on the move

Suzanne R. Pfeffer

Caveolae deliver simian virus 40 (SV40) to a new compartment called a caveosome, where the virus is sorted for transport to the smooth endoplasmic reticulum (ER). This pathway bypasses other endocytic organelles, and early steps may be shared by lipid rafts and certain glycosylphosphatidylinositol (GPI)-linked proteins.

Lipid rafts and caveolae have attracted significant attention as foci for signalling events at the plasma membrane and as mediators of protein sorting in the Golgi complex[1–4]. Caveolae are membrane domains that are enriched in GPI-linked proteins, glycosphingolipids, cholesterol and caveolin proteins. Caveolins themselves are fascinating proteins that associate with lipid rafts at the plasma membrane and are important for transporting GPI-linked proteins and cholesterol[1–4]. They bind to cholesterol and glycosphingolipids and form oligomers in response to cholesterol. Caveolae were first identified in endothelial cells and are present in most cell types. Although the transport of caveolae has been a somewhat controversial subject, the identification of caveolin as a key constituent of this domain has afforded a closer look at the dynamics and internalization of caveolar membranes.

Two recent reports have now revealed new pathways taken by caveolin and GPI-linked proteins. On page 473 of this issue, Pelkmans *et al.*[5] describe the internalization of non-enveloped SV40 virus, and identify a new endocytic compartment, the caveosome, that receives caveolin-decorated vesicles from the plasma membrane. Nichols *et al.*[6], by monitoring GPI-linked proteins that are thought to be internalized by caveolae, have detected a similar pathway; they describe the continuous cycling of GPI-linked proteins between the cell surface and the Golgi complex. SV40 leaves caveosomes and is delivered to the ER, and although it remains to be seen whether internalized GPI-linked proteins also use caveosomes as an intermediate station en route to the Golgi complex, both studies have revealed endosome-independent routes for endocytic transport to the ER and Golgi, respectively (Fig. 1).

In contrast to many enveloped viruses that use clathrin-coated vesicles to enter cells, SV40, a non-enveloped DNA virus, is internalized through caveolae[7,8] and uses major histocompatibility complex (MHC) class I antigens as receptors[9]. Kartenbeck *et al.*[7] noted that SV40 enters cells through small (60 nm) uncoated vesicles that excluded fluid-phase tracers. They later observed virions in tubular structures that contained multiple virus particles, and after 1–2 h viruses were present in tubular extensions of the ER. Members of the same group[5] have now used video-enhanced, dual-colour fluorescence to visualize the uptake of individual SV40 particles into live cells. The authors labelled SV40 directly with Texas Red and examined its entry into cells expressing caveolin tagged with green fluorescent protein (GFP).

Viruses first became trapped within caveolin-containing structures at the cell surface that would suddenly disappear from the plane of focus, which is consistent with internalization. The authors did not detect caveolin internalization in the absence of

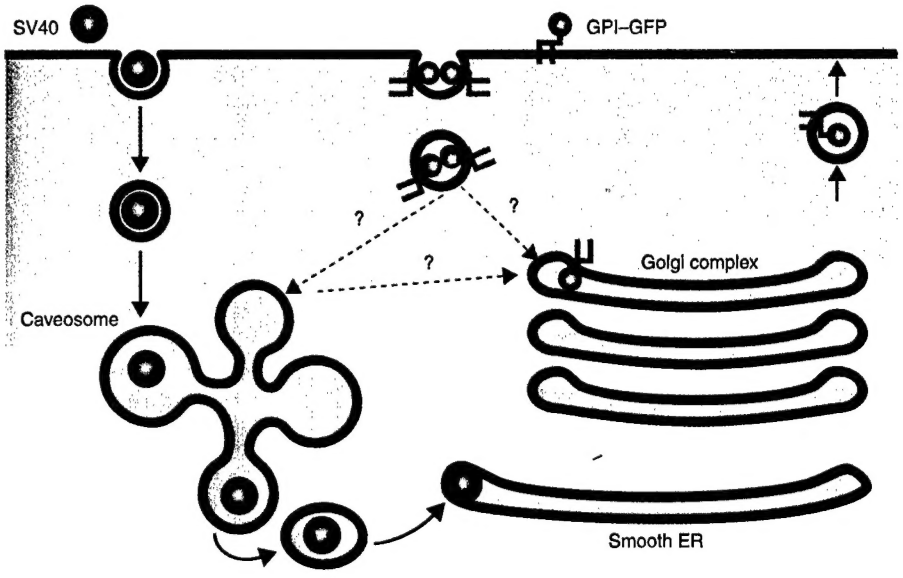


Figure 1 **SV40 enters cells by binding to receptors and subsequent capture by caveolae. Small vesicles form and deliver the virus to caveosomes. After several hours, tubules containing SV40 pinch off and deliver SV40 to the smooth ER. Certain GPI-linked proteins may also be internalized by caveolae and can be detected in endocytic compartments that lack endosome markers. They are then transported to the Golgi complex and cycle back to the plasma membrane, possibly in constitutive secretory vesicles. It is not known whether GPI-linked proteins also enter caveosomes.**

CELL BIOLOGY:

Do Centrosome Abnormalities Lead to Cancer?

Jean Marx

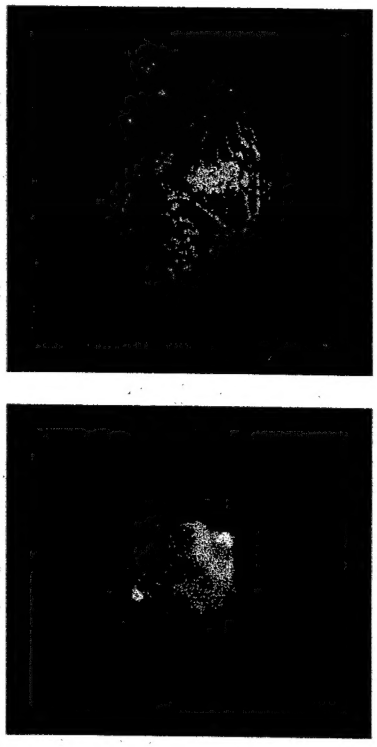
Evidence suggests that at least some cancers arise because centrosome malfunction causes chromosome damage and missorting

It may be small and inconspicuous, but the structure called the centrosome plays a big role in the cell. One key duty: helping to organize the mitotic spindle--the collection of protein filaments that pull the duplicated chromosomes apart during cell division, thereby ensuring that the two daughter cells each get a complete set. Without the centrosome, normal division of human cells could not occur. But accumulating evidence hints that this structure has a dark side as well. When the centrosome malfunctions, cancer may result.

Researchers have known for decades that cancer cells are rife with chromosomal abnormalities. Some cells lack one or more chromosomes, for example, while having extra copies of others. "Virtually every cancer cell has an abnormal chromosome complement, whereas virtually every normal cell has the [normal] diploid number," says cancer researcher Bert Vogelstein of Johns Hopkins University School of Medicine. The conventional wisdom has been that this aneuploidy, as it's called, is a late event in cancer development--the result of all the other disruptions in cancer cells. But now, "more and more it's coming out that [aneuploidy] is an early change and may be driving malignancy," says Vogelstein, whose own work has been pointing in that direction.

Contributing to this new view of aneuploidy is the realization that many types of cancer cells have abnormalities in their centrosomes--in particular, the cells often have extra copies. The supposition now is that the extra centrosomes lead to chromosome missorting and damage, thus causing the aneuploidy. Aneuploidy, in turn, may result in the loss of tumor suppressor genes or the gain or activation of cancer-causing oncogenes. "Once you have multiple centrosomes, that could increase the error rate [in chromosome replication and sorting], and those errors could be very dangerous," says centrosome researcher Greenfield Sluder of the University of Massachusetts Medical School in Worcester.

Researchers caution that the progression from centrosome derangements to aneuploidy to cancer isn't yet firmly established. Moreover, centrosome abnormalities likely aren't the only route to aneuploidy. For example, problems with the telomeres--the protective structures capping the ends of the chromosomes--have been implicated in the aneuploidy seen in some cancer cells. And two reports in the April issue of *Nature Cell Biology* suggest that mutations in a gene called *APC* , which are known to predispose to colon cancer, contribute to the chromosomal instability associated with that malignancy. But if centrosome abnormalities underlie at least some of the aneuploidy seen in cancer, they might be useful as diagnostic or prognostic indicators to help clinicians distinguish highly malignant cancers from those that are less dangerous. They might also point to possible new therapeutic strategies aimed at restoring normal centrosome function.



Centrosomal chaos. The mouse mammary cancer cell (top) has multiple centrosomes (red) and has generated four sets of spindle microtubules (green), which will lead to abnormal partition of the chromosomes (blue) in the daughter cells. At right is a normal dividing mammary epithelial cell.

CREDIT: W. BRINKLEY AND T. GOEPFERT

Hints of centrosome involvement

Early in the 20th century, a prescient microscopist named Theodor Boveri suggested that centrosome malfunction might lead to cancer. But the idea was more or less forgotten until about 5 years ago. At that time, Kenji Fukasawa, then in George Vande Woude's lab at the Frederick Cancer Research and Development Center in Frederick, Maryland, and colleagues found that cells lacking a critical tumor suppressor gene, known as *p53* , have multiple centrosomes instead of the normal one or two.

In work described in the 22 March 1996 issue of *Science* (p. 1744), the researchers reported that in cell culture, this centrosome amplification apparently disturbs mitotic fidelity, causing the cells to end up with abnormal chromosome complements. Because *p53* 's loss or inactivation is thought to contribute to the development of many human cancers, these findings suggested a new way that lack of a functional *p53* gene might lead to cancer: by disturbing centrosome function and thereby generating aneuploidy.

Two years later, a team led by Dennis Roop and William Brinkley of Baylor College of Medicine in Houston, Texas, produced evidence that this can in fact happen, at least in an animal cancer model. When the researchers blocked *p53* activity in skin cells of living mice, those animals developed skin cancers when exposed to carcinogenic chemicals much more readily than controls did, and the centrosomes were amplified in the cells of 75% of the tumors. (The results appeared in the July 1998 issue of *Oncogene* .)

At about the same time, Jeffrey Salisbury of the Mayo Clinic Foundation in Rochester, Minnesota, and his colleagues found centrosome abnormalities in the cells of human breast cancers, and Stephen Doxsey and German Pihan of the University of Massachusetts Medical School detected them in most of the common human cancers, including breast, prostate, lung, colon, and brain. In addition to extra centrosomes, the researchers saw oversized centrosomes and some that contained more than the normal amounts of phosphate groups. "When we first looked at breast tumors, it was striking how unusual the centrosomes were. They stuck out like a sore thumb," Salisbury says.

But could centrosomal defects such as these actually cause aneuploidy and thus possibly contribute to the development of the cancers? Some hints that they might came from Doxsey's work, which showed a strong correlation between centrosome abnormalities and chromosome instability, and from Thomas Ried and his colleagues at the National Cancer Institute in Bethesda, Maryland. As reported in the February 2000 issue of *Genes, Chromosomes and Cancer* , when the Ried team looked at cultured lines of human colorectal cancer cells, they detected aneuploidy only in those cell lines that also displayed centrosome abnormalities. Ried cautions, however, that this work simply shows a correlation between the centrosome and chromosome abnormalities: "Causality has not been established."

How centrosomes might go awry

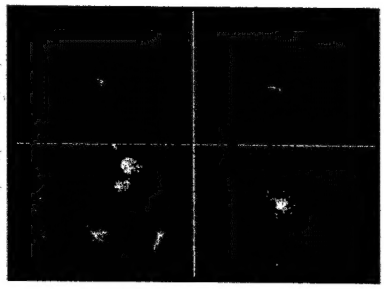
Whatever their role in cancer, if any, researchers want to know what causes the centrosomal abnormalities. They have unearthed several intriguing possibilities. Fukasawa's earlier findings pointed to loss or inactivation of the *p53* tumor suppressor gene as one possibility. More recently, Fukasawa, who is now at the University of Cincinnati College of Medicine, has been working out just how that loss leads to centrosome amplification.

As shown 2 years ago by Sluder and Massachusetts colleague Edward Hinchcliffe, and independently by Tim Stearn's team at Stanford University, the activity of a kinase enzyme called CDK2 is needed for centrosome replication (*Science* , 5 February 1999, pp. 770 and 851). Because CDK2 activity is also needed to drive cells through the division cycle, this helps ensure that the centrosome replicates only once and at the right time. The Fukasawa team now has evidence that CDK2 controls centrosome replication by tacking a phosphate group onto a centrosome protein called nucleophosmin, causing it to leave the centrosome. Nucleophosmin's departure then initiates centrosome duplication, Fukasawa says. The *p53* gene comes into this picture because its protein product, working through another protein called Waf-1, inhibits CDK2. Thus, *p53* 's absence allows the centrosome to replicate when it shouldn't and accumulate extra copies.

Inappropriate activity of other kinases may also lead to the centrosome abnormalities seen in cancer cells. One of these is a so-called aurora kinase, discovered about 15 years ago in the fruit fly *Drosophila melanogaster* by David Glover of the University of Dundee, Scotland, and his colleagues. They found that when the gene encoding this kinase is mutated, mitosis is disrupted in fly cells, apparently because the kinase is needed to separate the duplicated centrosome before cell division.

The first clue that an aurora kinase might be involved in human cancers came about 3 years ago. Two groups, one led by Brinkley and Subrata Sen of the University of Texas M. D. Anderson Cancer Center in Houston and the other by James Bischoff and Gregory Plowman of SUGEN Inc., in Redwood City, California, found an aurora kinase gene in a region of chromosome 20 that is amplified, or present in multiple copies, in many colon, breast, and other tumors. Presumably as a result of the amplification, the protein itself was present in the cancer cells in abnormally high concentrations.

To see whether the elevated aurora kinase levels actually cause the centrosome defects, and possibly the cancers, the Texas team genetically engineered noncancerous cells that had the normal one or two centrosomes to overproduce the enzyme. As a result, Brinkley says, the cells "produced multiple centrosomes, became aneuploid, and [displayed] other characteristics of transformed [cancerous] cells."



Too much of a good thing? Centrosomes from cancer cells (bottom) nucleate the formation of many more microtubule fibers than do centrosomes from normal cells (top).

CREDIT: W. L. LINGLE AND J. L. SALISBURY

Abnormalities in the proteins that make up the centrosome structure have also been linked to cancer. For example, Doxsey and his team have found that concentrations of a centrosome protein called pericentrin are higher than normal in prostate and other cancers. And, similar to the situation with aurora kinase, when the researchers genetically engineered normal cells to overproduce pericentrin, the cells developed extra centrosomes. Says Doxsey: "We can create in vitro what's going on in tumor cells."

But questions remain ...

Many questions must still be answered to firm up the link between centrosome abnormalities and cancer development. For example, if the abnormalities are causative, one might expect to find mutations in the genes for centrosome structural or regulatory proteins in tumors. Aside from the amplification of the aurora kinase gene, none has been found so far, although Mark Winey, who studies centrosomes in yeast, thinks "it's just for want of looking."

Another question is how cells with centrosomal abnormalities manage to divide and survive at all. Normally, a variety of so-called checkpoints ensure that a cell doesn't divide if its DNA is damaged or abnormal. So "how does a cancer cell become a virtual dividing machine in the presence of all those centrosomes?" Brinkley asks.

One possibility is that multiple centrosomes coalesce at the poles of a dividing cancer cell. That way, instead of having multiple spindle poles, the cell would have just two functional poles that partition the chromosomes equally. Brinkley, Salisbury, and others have detected such structures in some cancer cells.

Perhaps the biggest question is when in cancer development the centrosomal abnormalities and aneuploidy arise. If they are driving cancer formation, as opposed to being a consequence of it, "they should be present not only in bad tumors, but in early ones," Doxsey says. Although the case isn't airtight, the researchers do have some evidence that the abnormalities are present in early tumors.

For example, in work that's not yet been published, Brinkley and his colleagues treated young mice with a carcinogen that induces breast cancer and then periodically examined samples of mammary gland cells to see when extra centrosomes appeared. That turned out to be just 90 days after the carcinogen treatment began, when the tissue showed precancerous changes but had not formed full-fledged tumors, Brinkley says.

Salisbury has also detected centrosomal abnormalities in ductal carcinomas in situ--an early stage of human breast cancer. And Doxsey and his colleagues report in the March issue of *Cancer Research* that they have found the abnormalities in a variety of early cancers, including 15% to 20% of prostate cancers.

Doxsey and his colleagues are now exploring whether centrosome abnormalities, or concentrations of the centrosome protein pericentrin, can be used to help clinicians assess the aggressiveness of prostate tumors. Most of the tumors that are detected early will grow so slowly that they won't be a danger to the patient, but right now they can't be distinguished from the fast-growing ones. As a result, many men may have their prostate glands removed unnecessarily.

Doxsey notes that the percentage of early prostate tumors in which he found the centrosome abnormalities is about the same as the percentage of dangerous tumors. His team's work also shows a correlation between the degree of centrosome abnormality and cytological indicators of tumor seriousness. If those abnormalities can be used as a prognostic indicator, the tiny centrosome may prove a big help to patients with prostate and other cancers.

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